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(54) Title: ENHANCED PRODUCTION OF SECONDARY METABOLITES IN THE PRESENCE OF ORGANIC ADDITIVES

(57) Abstract: A method for improving the level of production and an enhancement of the component profile and chemical diversity of secondary metabolites produced by organisms is disclosed. The method comprises the addition of at least one organic additive to the growth medium inoculated with the organism, within which the secondary metabolite or secondary metabolites are produced. Suitable organic additives include dimethyl sulfoxide, dimethyl sulfone and ethanol; dimethyl sulfoxide is particularly preferred. The organic additives of the present invention are used at a concentration within a range of 0.1 % to 10 % (v/v) in the growth medium, preferably within the range of 0.5 to 7.5 %, and most preferably within the range of 1.0 to 5 %.

represented within this group of natural products have provided a valuable resource in the continuing search for novel chemical structures that may be of clinical and commercial importance.

The pharmaceutical industry, in particular, has discovered and developed a large number of secondary metabolites useful in the prevention, amelioration and cure of human disease. Secondary metabolites also have provided "lead structures" that can be chemically modified to diminish their toxicity and to improve their specificity and pharmacokinetic traits. These natural products and their semi-synthetic derivatives have found application as antibacterial, antifungal, antiviral, antihelminthic and anticancer agents. Other secondary metabolites have been discovered to have immunosuppressive traits and anticholesterolemia properties.

In light of the importance and proven utility of secondary metabolites, extensive environmental screening programs have been established to detect organisms that elaborate new and useful natural products. These searches have been extended to the oceans and jungles, and even to the bark of trees, of ever-more remote environmental niches of the world. Not surprisingly, many of the organisms detected exhibit unusual and rather complex requirements for growth in the laboratory. In addition, historical experience has demonstrated that the synthesis of secondary metabolites is very sensitive to environmental conditions and is also possibly influenced by one or more, usually unidentified, molecular signals generated within that environment by the growth of the organism. Accordingly, production of a secondary metabolite by a cultured organism has been observed to be remarkably and unpredictably sensitive to the composition of the medium within or upon which that organism is grown. Furthermore, it has been observed that, even under "optimal" laboratory conditions, the level of secondary metabolite produced by a wild-type organism is usually extremely low. Therefore, there is always a concern that valuable molecules may be overlooked simply because the level of production of that secondary metabolite did not rise to the threshold level required for its detection.

Discovery programs, designed to detect and identify novel chemical structures elaborated by newly-isolated or newly-constructed organisms, frequently employ a relatively large number of different growth media to increase the probability that at least one will support synthesis of a secondary metabolite having a desired activity. Nevertheless, such an approach will fail to detect those secondary metabolites whose production level does not reach the threshold of detection.

In those instances in which a potentially useful metabolite has been detected, a sufficient amount of that molecule is isolated to evaluate its specificity, selectivity,

efficacy, and, possibly its toxicity, in addition to a determination of its chemical structure. Production of material for these studies is usually done using the identical isolate and growth medium used where the signal was first detected, even though it may not be efficient, to insure that the same activity is followed throughout.

If these pilot studies indicate that the secondary metabolite is novel and useful *per se*, or may be valuable as a core structure or lead compound that can be further modified chemically or biologically, a development program is initiated to increase the level of production of that metabolite. Many approaches, and combinations of approaches have been employed, including those relying upon random mutagenesis and screening, directed screening, molecular biology and genetic engineering. However, although each of these methods may ultimately provide genetically superior producers of one or more secondary metabolites, that full genetic potential will not be realized unless the growth medium supporting production of that metabolite has also been optimized.

Similar problems and obstacles are presented by alternative methods for the generation of novel compounds, including mutasynthetic, co-synthetic, biotransformation, and recombinant DNA procedures. Often, the level of production of the newly generated secondary metabolite is extremely low. This is particularly true in those instances involving heterologous biosynthetic intermediates that may be only poorly recognized and bound by one or more active sites required for the biosynthesis of the final compound.

Methods for enhancing production of secondary metabolites are needed for the analysis of newly-discovered and newly-constructed organisms producing these molecules to allow the detection of the full spectrum of compounds synthesized by those organisms. Similarly, improved methods for the production of identified secondary metabolites are required to generate adequate amounts of those compounds to facilitate their isolation, characterization and evaluation. Moreover, media development is often required to realize the genetic potential of an organism producing a secondary metabolite of interest, regardless of the method by which the strain was isolated, selected or constructed. Furthermore, approaches that increase the level of production of a secondary metabolite are needed to minimize the cost of production of these materials on a commercial scale.

Therefore, although there are a number of approaches that have been developed and used to improve the production of secondary metabolites, none of these is generally applicable to each of these phases: discovery, development and commercial production of a secondary metabolite. Accordingly, there is still a need for a generally-applicable method that will increase the level of production of secondary metabolites of interest.

3. SUMMARY OF THE INVENTION

The object of the present invention is to manipulate the composition of the cell culture media to obtain an increase in the production of desirable and useful secondary metabolites.

The invention provides in one embodiment, a method for increasing the production of a secondary metabolite by an organism, comprising culturing the organism in a medium comprising an amount of at least one organic additive, which amount is sufficient to increase the production of the secondary metabolite.

Preferably, the method of the invention comprises increasing the production of useful metabolites by culturing microbial and plant cells in a culture medium suitable for growth containing at least one of the organic additives dimethyl sulfoxide (DMSO), ethanol or dimethyl sulfone, the additive being present at a concentration between about 0.1% and about 10% v/v, preferably between about 0.5 and 7.5% v/v, and more preferably between about 1 % and about 5% v/v.

In another embodiment, the invention provides a culture medium for production of secondary metabolites by organisms, comprising a nutrient medium capable of supporting the growth of the organisms and at least one organic additive at a concentration between about 0.1% to about 10% v/v. The preferred organic additives are dimethyl sulfoxide (DMSO), ethanol and dimethyl sulfone.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the variation of the secondary metabolite tetracenomycin (TCMC) produced by *Streptomyces glaucescens* grown in TSB medium in the presence of various concentrations of DMSO.

Figure 2 is a graph showing the variation of the secondary metabolite chloramphenicol produced by *Streptomyces venezuelae* grown in soy flour medium in the presence of various concentrations of DMSO.

Figure 3 is a graph showing the variation of the secondary metabolite chloramphenicol produced by *Streptomyces venezuelae* grown in GNY medium in the presence of various concentrations of DMSO.

Figure 4 is a graph showing the variation of the secondary metabolite thiostrepton produced by *Streptomyces azureus* grown in R5 medium in the presence of various concentrations of DMSO.

Figures 5a and 5b are graphs showing the variation of low molecular weight secondary metabolites produced by transformant S10H10 of *Streptomyces lividans* grown in R5 medium containing 25 µg/ml apramycin, in the presence and absence of DMSO, respectively.

Figures 6a and 6b are graphs showing the variation of low molecular weight secondary metabolites produced by transformant 446-S3-102H4 of *Streptomyces lividans* grown in R5 medium containing 25 µg/ml apramycin, in the absence and presence of DMSO, respectively.

Figures 7a and 7b are graphs showing the variation of low molecular weight secondary metabolites produced by transformant 446-S3-81 D3 of *Streptomyces lividans* grown in R5 medium containing 25 µg/ml apramycin, in the absence and presence of DMSO, respectively.

Figure 8 is a graph showing the variation of the secondary metabolite tetracenomycin (TCMC) produced by *Streptomyces glaucescens* grown in TSB medium in the presence of ethanol (EtOH).

Figure 9 is a graph showing the variation of the secondary metabolite tetracenomycin produced by *Streptomyces glaucescens* grown in R5 medium in the presence of ethanol.

Figure 10 is a graph showing the variation of the metabolite chloramphenicol produced by *Streptomyces venezuelae* grown in GNY medium in the presence of dimethyl sulfone.

5. DISCLOSURE OF THE INVENTION

The present invention relates to an improved process for the production of secondary metabolites by cultured organisms. The present invention is directed both to methods through which the level of production of a secondary metabolite is increased and to methods through which the component profile of secondary metabolites produced by a cultured organism is enhanced and diversified.

The method of the invention involves culturing microbial and plant cells in a nutrient medium capable of supporting their growth and viability, and adding to the medium a sufficient quantity of at least one organic additive to increase the production of secondary metabolites of interest. In preferred embodiments of the invention, the organisms are fungi, bacteria, or plants. The culture medium for the organisms is medium which is known to support the growth and viability of the particular species of organisms. Such media are well

known in the art, and several types of media are described in the examples below. Media and conditions for growing organisms can be found in Demain A. L. and N.A. Solomon (eds.) (1986) Manual of Industrial Microbiology and Biotechnology, American Society for Microbiology, Washington D.C., the disclosure of which is hereby incorporated by reference.

The term "organic additive" as used herein includes organic molecules having a molecular weight of less than about 250, wherein the organic molecule comprises at least one heteroatom selected from the group consisting of sulfur, oxygen and combinations thereof. Organic additives of the present invention include, but are not limited to dimethylsulfoxide (DMSO), dimethly sulfone and lower alcohols including methanol, ethanol, propanol, isopropanol, butanol, n-butanol, iso-butanol, t-butanol, amyl alcohol and iso-amyl alcohol, and combinations thereof. DMSO, which is a natural product and exhibits low toxicity, is the preferred additive. In another embodiment of the present invention, organic additives include organic molecules having a molecular weight of less than about 250, wherein the organic molecule consists essentially of carbon, hydrogen and at least one heteroatom selected from the group consisting of sulfur, oxygen and combinations thereof.

The organic additive is preferably added to the culture of organisms at the time of innoculation, but it can be added at any time during the culture period. The concentration of the organic additive added does not take into account, for example, the production of ethanol by an organism synthesizing one or more secondary metabolites.

As used herein, the phrase "in a nutrient medium" comprises "on a nutrient medium" as well; that is, it includes growing one or more organisms on media solidified by the inclusion of a solidifying agent, for example, agar or agarose, to produce a "semi-solid" nutrient medium.

The invention is applicable to any type of culturing or fermentation system or process, including batch, fed-batch, and continuous fermentation processes. The invention may also be applied to processes for the production of one or more secondary metabolites using immobilized cells. In addition, the invention may be used to increase the production of one or more secondary metabolites, or to enhance the component profile and chemical diversity of secondary metabolites elaborated by an organism grown on semi-solid media, including media comprising, for example, agar or agarose, which may be used as solidifying agents.

The invention may also be used for the production of secondary metabolites by marine organisms grown in media comprising a relatively high salt content.

The optimal concentration of the organic additive in the culture medium will depend on several factors, including the nature of the organic additive, the strain of organism being cultured, as well as the particular culture medium used. The optimal concentration for a particular organism can easily be determined using assays similar to the ones outlined herein. However, the optimal concentration will generally be found within the range of 0.1 to 10% v/v, and preferably within the range of 0.5 to 7.5% v/v, and most preferably within the range of 1.0 to 5% v/v.

The invention also contemplates a complete culture medium for producing secondary metabolites from organisms, wherein the culture medium comprises an organic additive. Preferably, the organic additive is present in the culture medium at concentrations ranging from about 0.1% to about 10% v/v. Examples of such culture media are provided in Section 6.

The methods and compositions of the invention can be used in conjunction with other culture medium additives which promote growth of organisms and production of secondary metabolites.

The term "organisms," as used in herein includes, bacteria, fungi and plant cells. Bacteria include, but are not limited to the *Actinomycetes* family and, particularly, species of *Streptomyces*, including but not limited to *Streptomyces hygroscopicus*, *Streptomyces halstedii*, *Streptomyces erythrae* (now known as *Saccharopolyspora erythrae*), *Streptomyces mediterranei*, *Streptomyces aureofaciens*, *Streptomyces venezuelae*, *Streptomyces spheroides*, *Streptomyces cinnamonensis*, *Streptomyces albus*, *Streptomyces caespitosus*, *Streptomyces avermitilis*, *Streptomyces parvulus*, *Streptomyces antibioticus*, *Streptomyces azureus*, *Streptomyces griseus*, *Streptomyces clavuligeris*, *Streptomyces lavendulae*, *Streptomyces lincolnensis*, *Streptomyces curacoi*, *Streptomyces fradiae*, *Streptomyces rimosus*, *Streptomyces peucetius*, *Streptomyces lividans*, *Streptomyces sp.* (ATCC accession number 55656), and *Streptomyces roseosporus*.

Non-*Streptomyces Actinomycetes* include, but are not limited to species of *Actinomadura, Actinoplanes*, *Actinosporangium*, *Actinosynnema*, *Ampullariella*, *Chainia*, *Dactylosporangium*, *Kibdelosporangium* (including *Kibdelosporangium sp.* ATCC accession number 53771), *Kitasatosporia*, *Kitasatoa*, *Microellobosporia*, *Micromonospora* (including for example, *Micromonospora purpurea*, *Micromonospora rosaria*, *Micromonospora chalcea*, and *Micromonospora echinospora*), *Microtetraspora*, *Nocardia* (including *Nocardia lactamdurans*), *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Streptoalloteichus*, *Streptosporangium*, *Streptoverticillum*, *Thermoactinomyces*, and *Thermomonospora*.

profile" and the term "complex" are used interchangeably to refer to the mixture of secondary metabolites that are co-produced by an organism.

As used herein, an enhanced component profile refers to a component profile, obtained from an organism cultured in the presence of an organic additive, which has at least one component or peak that was not detected when the organism was cultured in the absence of the organic additive. Applicants believe, without being bound to that belief, that the new peaks or components disclosed, for example, in Figures 5, 6 and 7 may represent an increase in the level of production of one or more secondary metabolites, in the presence of the organic additive, which now surpasses the threshold of the detection method employed. Alternatively, Applicants believe, without being bound to that belief, that the new peaks or components may be a consequence of the induction of expression of one or more genes in the organism, the products of which comprise activities that result in the synthesis of secondary metabolites that are not produced in the absence of the organic additive.

Therefore, an enhanced component profile may comprise an increase in the level of one or more peaks or the presence of a previously-undetected peak. Furthermore, an enhanced component profile may also be characterized by the absence or diminution of a previously-detected peak, especially in those instances in which the molecules represented by that peak interfere with the detection or purification of a secondary metabolite of interest. An enhanced component profile also comprises one exhibiting a combination of more than one of these phenomena.

As used herein, growth of an organism in a culture medium for the production of a secondary metabolite includes those processes, known in the art, referred to as cosynthesis, mutasynthesis, and biotransformation. It also includes, as demonstrated in Examples V, VI and VII, the synthesis of secondary metabolites by recombinant organisms.

Cosynthesis, as used herein, comprises the growth of a plurality of organisms in a culture medium whereby at least one secondary metabolite is synthesized.

Mutasynthesis, as used herein, comprises growth of at least one organism, wherein the organism carries a mutation preventing the synthesis of a first secondary metabolite that its non-mutant parent can synthesize, in a culture medium comprising a second metabolite, whereby that second metabolite is modified by the mutant strain.

Biotransformation, as used herein, comprises the growth of at least one organism in a culture medium comprising a metabolite, whereby the metabolite is modified. An example of a secondary metabolite produced by biotransformation is disclosed in U.S. Patent No. 5,468,771, which discloses the formation of a cholesterol-lowering compound by

cultivating *Streptomyces cyanus* (ATCC 5214) in a culture medium comprising the precursor molecule to be modified. A similar procedure is disclosed in U.S. Patent No. 5,194,377 to Schwartz *et al.*, where the growth medium comprised amino acid analogues that were incorporated into the antifungal secondary metabolites produced by *Mycoleptodiscus atromaculans*.

Recombinant processes, as used herein, include the growth of at least one recombinant organism in a culture medium for the production of at least one secondary metabolite. Recombinant processes comprise those performed with an organism carrying at least one gene, which has been modified by or incorporated within the organism using recombinant DNA tools and methods which are well known to those of ordinary skill in this art. Examples of such processes are disclosed in U.S. Patent Nos. 5,843,718; 5,830,750; 5,712,146 and 5,672,491 to Khosla *et al,* U.S. Patent No. 5,824,485 to Thompson *et al.*, U.S. Patent No. 5,783,431 to Peterson *et al.*, U.S. Patent No. 5,882,883 to Egel-Mitani *et al.*, U.S. Patent 5,462,862 to Groenen *et al.*, U.S. Patent No. 5,479,912 to Sherman *et al.*, and U.S. Patent No. 5,710,032 to Piepersberg *et al.*.

For example, U. S. Patent Nos. 4,208,403 (reissued as RE 32,333) and 4,331,594 (reissued as RE 32,455) which are incorporated herein by reference in their entireties, disclose the production, isolation, and identification of A-21978 antibiotic complexes by *Streptomyces roseosporus*, and, in particular the A-21978C complex of antibiotics. The individual components of the A-21978C complex are cyclic polypeptides carrying a fatty acid moiety acylated to the amino terminus of the cyclic polypeptide.

U. S. Patent Nos. 4,800,157 and 4,885,243, which are incorporated herein by reference in their entireties, disclose, respectively, an improved isolate of *Streptomyces roseosporus*, and an improved process for the production of the A-21978C complex of antibiotics, including the n-decanoyl derivative of A-21978C.

More specifically, the term "daptomycin" refers to the n-decanoyl derivative of A-21978C$_0$ type antibiotic. "Daptomycin" is synonymous with LY 146032. An exemplary process for production of daptomycin is as follows. *Streptomyces roseosporus* is fermented with a feed of n-decanoic acid, as disclosed in United States Patent No. 4,885,243, with the modification that the decanoic acid feed is kept at the lowest levels possible without diminishing the overall yield of the fermentation. In a preferred embodiment, the residual decanoic acid is maintained at less than 50 parts per million (ppm) during aerobic fermentation. In a more preferred embodiment, the residual decanoic acid is maintained between one and 20 ppm during the aerobic fermentation. In an even more preferred embodiment, the residual decanoic acid is maintained at approximately ten ppm

during the aerobic fermentation. In a preferred embodiment, the concentration of residual decanoic acid is measured throughout the fermentation and the feed level of decanoic acid is adjusted to continuously keep the residual decanoic acid levels within the preferred parameters. The prior art does not describe the *in situ* specific and low residual constant decanoic acid concentration required to achieve optimal expression of daptomycin containing lower levels of impurities.

Therefore, the process disclosed herein will also facilitate the detection and isolation, for example, of new secondary metabolites that are structurally related to known chemical entities. Similarly, secondary metabolites may be detected using the methods of the present invention, which were not known to be produced by prior art processes. The process of the present invention will also facilitate the detection, isolation and characterization of novel chemical structures by providing increased production levels and enhanced component profiles for secondary metabolites. Mixtures of secondary metabolites comprising new activities or new peaks may be fractionated chromatographically, for example by high-performance liquid chromatography (HPLC). Appropriate fractions of the profile may be collected, concentrated as necessary and further purified using methods well known to those of ordinary skill in the art. These could include a separation step based upon the size of the molecule, employing, for example, chromatographic separations on Sepharose® columns. Determination of the structure of the novel compound isolated would be established using data provided by a number of analytical methods that are well known to those of ordinary skill in the art. Diode array detectors could be used to obtain concurrent absorption spectra for compounds undergoing HPLC separation; liquid chromatographic separations can be directly linked to mass-spectral determinations to provide characteristic molecular fragmentation patterns; and a number of NMR experiments may also be performed, as necessary. In combination, these analytical methods may be applied to novel secondary metabolites, which have been provided in greater quantity than heretofore possible by the present production process, to establish their chemical structure.

The methods of the present invention can be applied to those discovery approaches in which newly-discovered or newly-constructed organisms are grown in or on a number of different media, assembled empirically over time, such that at least one set of conditions will be suitable for the synthesis of secondary metabolites at a detectable level. In these approaches, testing of each organism will result in the generation of a correspondingly large set of samples to be examined for compounds exhibiting useful properties. Each of these samples is then tested in a wide array of activity screens

employing a number of assays of varying degrees of sensitivity and specificity, generating a signal where the material examined has a desired trait. Therefore, supplementation of the media used, whether it be liquid and semi-solid, with the organic additives disclosed herein is expected to increase the level of production of secondary metabolites and to enhance the component profile of those secondary metabolites elaborated by the organisms screened in these discovery programs.

High-throughput screens can be designed within which a population of organisms, which may include newly-isolated or newly-constructed strains, are distributed onto semi-solid media or into individual wells containing growth media. Subsequently, secondary metabolites produced under these conditions are screened for biological activity by sampling the growth media surrounding the colony. Alternatively, for example, an indicator organism may be overlaid onto the semi-solid medium upon or within which the secondary-metabolite-producing organisms are growing. The indicator may simply be an organism sensitive to an antibiotic produced as a secondary metabolite or the indicator can be an organism that will generate, or has been genetically engineered to generate, a detectable signal in the presence of a compound having a desired activity. Examples of the latter include indicator strains, isolated or constructed, which will generate a detectable signal in the presence of an agonist or an antagonist of receptor binding. Such systems are only limited by the imagination and creativity of the investigator and many examples are known to those of ordinary skill in the art. Non-limiting examples of screening methods are found in the following representative patents: U.S. Patent Nos. 5,780,294 to Siehl *et al.*, 5,914,323 to Mundy *et al.*, 5,948,612 to Bascomb *et al.*, and publications: Stockwell *et al.* (1999) *Chem Biol* 6(2): 71-83; Kunkel *et al.* (1997) *Anticancer Drug Res* 12(8): 659-670; Shawar *et al* (1997) *Antimicrob Agents Chemother* 41(3): 570-574; Wu *et al.* (1997) *Anal Biochem* 245(2): 226-230; Slack *et al.* (1989) *Biotechniques* 7(10): 1132-1138; Bartus *et al* (1984) *Antimicrob Agents Chemother* 25(5): 622-625.

Application of the methods of the present invention, supplementing the media employed with one or more of the organic additives disclosed, is expected to increase the probability of detecting interesting secondary metabolites both by increasing the level of production thereof, and by enhancing the component profile of secondary metabolites elaborated by the population of producing organisms examined.

Similarly, the methods of the present invention can be applied to those processes initiated once a potentially-useful molecule has been detected, and a sufficient amount of that compound is to be produced to verify preliminary activity data, to provide a preliminary evaluation of the compound, and to permit a determination of its structure. The

amount of material required for these pilot and range-finding experiments is often considerable, when compared to the level of production of the secondary metabolite by the organism, and can create a serious impediment to development of a secondary metabolite. Accordingly, supplementation of the growth media with one or more organic additives is expected to facilitate such work by increasing the level of production of the secondary metabolite of interest or by inducing the production of that metabolite.

When justified by the results of pilot testing, the level of production of a secondary metabolite of interest can be improved by carrying out a coordinated development program involving both medium optimization and strain improvement. In these instances, the level of production of a secondary metabolite by an organism can be improved using, for example, empirical methods, directed procedures, molecular biology, genetic engineering, and combinations of these methods in one or more stages of overall process improvement.

Empirical procedures for improving the production of secondary metabolites were developed, historically, and generally rely upon random mutagenesis of the producing organism coupled with, essentially, trial-and-error media development. This approach requires little information concerning the organism, the biosynthetic pathway involved or even the structure of the secondary metabolite of interest. However, considerable labor and time may be required since large numbers of candidate strains must be evaluated to find those isolates that are, in fact, stable derivatives that are significantly improved producers of the secondary metabolite. Multiple rounds of mutagenesis and screening, which may be automated, are frequently necessary to develop a useful strain. Even with a demonstrably improved strain, media development must still be carried out to optimize production of the secondary metabolite. One of the liabilities of this approach is that it is very inflexible. The ultimate process developed, comprising the multiply-mutated strain and the corresponding medium optimized for production of the secondary metabolite of interest by that strain, is not generally applicable to a process for the production of any other secondary metabolite. However, incorporation of the methods of the present invention is expected to provide an increase in the level of production of at least one secondary metabolite in the production process developed in this manner.

More directed methods have been developed and applied in those instances where the structure of the secondary metabolite has been established and the biosynthetic pathway for its synthesis has been experimentally determined or is reasonably apparent. Occasionally, it has been possible to isolate mutants that are resistant to a metabolic inhibitor and consequently overproduce, for example, an amino acid that fortuitously, is the rate limiting substrate for synthesis of the secondary metabolite of interest. Such

approaches are effective within the limited number of instances in which they apply. Nevertheless, media development must still be carried out to optimize production of the secondary metabolite by the mutants isolated in this manner. In addition, information concerning the structure and biosynthetic process for the secondary metabolite must be available to enable the effective use of this approach. The level of production of a strain selected in this manner will, nevertheless, be strongly influenced by, if not determined by, the growth medium used for the production of the secondary metabolite. Accordingly, supplementation of that medium with one or more organic additives is expected to increase the level of production of that secondary metabolite.

A second directed approach can be employed if the secondary metabolite has antimicrobial activity or if an indicator organism has been isolated or constructed, which will provide a detectable signal in the presence of a desired secondary metabolite. Improved producers of that secondary metabolite may readily be identified from within a larger population grown on an agar medium by overlaying the producing colonies with a sensitive indicator organism. This method for improved production of a secondary metabolite is limited to those having antibiotic activity or those for which a signal-generating indicator organism is available; this approach also relies upon the availability of a suitable growth medium. Again, application of the methods of the present invention is expected to increase the level of expression of the secondary metabolite produced as well as to enhance the component profile of the secondary metabolites synthesized under these conditions.

More recent information concerning the molecular biology underlying the synthesis of secondary metabolites may be useful for developing processes leading to the improved production of secondary metabolites. For example, it has been discovered that diffusible, low-molecular weight molecules are involved in the regulation of secondary metabolism. One such molecule, designated A-factor, which is a gamma-butyrolactone derivative, binds to a specific receptor protein in *Streptomyces griseus* and triggers the expression of streptomycin biosynthetic pathway. Although the existence of A-factor homologues in a number of *Streptomyces* species suggests that gamma-butyrolactone derivatives and analogues may serve as autoregulatory, chemical signaling agents, these molecules do not appear to be generally recognized by other producers of secondary metabolites. Therefore, the identification and characterization of such a regulatory molecule involved in the synthesis of a secondary metabolite by one organism, will not be useful for enhancing the production of a different secondary metabolite by another, heterologous organism. However, in those instances where a signaling molecule has been identified and can be used to influence the production of a secondary metabolite, application of the

methods of this invention is expected to provide a further increase in the ultimate level of the secondary metabolite produced.

Second-site mutations have, in some instances, been shown to affect the level of synthesis of antibiotics. Shima *et al.* have demonstrated that specific streptomycin-resistant mutants of *S. lividans* (those leading to the substitution of glutamic acid or arginine for the lysine normally found at amino acid residue 88 of ribosomal protein S12) are markedly better producers of actinorhodin (Shima *et al.* (1996) *J. Bacteriol.* 178:7276-7284). These observations have been extended to include other examples of the acquisition of streptomycin resistance resulting in increased production of a secondary metabolite, including the production of fredericamycin by *Streptomyces chattanoogensis*, actinomycin by *Streptomyces antibioticus*, formycin by *Streptomyces lavendulae*, nucleoside antibiotic FR 900493 by *Bacillus cereus*, and pyrrolnitriin by *Pseudomonas pyrrocinia* (Hosoya *et al.* (1998) *J. Bacteriol.* 42: 2041-47).

Genetic engineering has also been shown to provide a basis for the precise and effective construction of strains that are genetically capable of producing improved levels of secondary metabolites. In one example, disruption of a negative regulator of antibiotic biosynthesis, *absA*, resulted the hyperproduction of actinorhodin and undecylprodigiosin, two of the secondary metabolites produced by the host strain, *Streptomyces coelicolor*. (D.J. Aceti and W.C. Champness, (1998) *J. Bacteriol.* 180 (12): 3100-3106.) Similarly, overexpression of a positive regulatory element, *ccaR*, *via* genetic engineering, resulted in a two- to three-fold increase in the production of cephamycin and clavulanic acid, two of the secondary metabolites synthesized by *Streptomyces clavuligerus*. (F.J. Perez-Larena, P. Liras, A. Rodriguez-Garcia and J.F. Martin (1997) *J. Bacteriol.* 179 (6): 2053-2059).

However, the application of genetic engineering to the overproduction of a secondary metabolite requires detailed knowledge of the structure, and sequence of the regulatory genetic elements, as well as the mechanism through which these elements control expression of biosynthetic genes involved in the production of the secondary metabolite of interest. Such approaches also rely upon relatively idiosyncratic methods and tools for the manipulation of genetic information within each producing organism. Consequently, genetic engineering approaches that improve the production of a secondary metabolite are precise and effective, but they depend upon the preliminary acquisition of a substantial amount of information and the improvements are applicable only to that organism or to the specific pathway that has been manipulated.

Nevertheless, media development will still be required in order to realize the biosynthetic potential of the genetically-engineered organism constructed for the synthesis of a given secondary metabolite. Again, application of the methods of the present invention is expected to increase the level of production and enhance the component profile of genetically engineered producers of secondary metabolites.

It is known that various species of the bacterial genus *Streptomyces* produce useful antibiotics such as chloramphenical, daptomycin, tetracenomycin, and thiostrepton, and other secondary metabolites. In addition to antibiotics, organisms produce other secondary metabolites which are industrially useful, including bacterial and fungal secondary metabolites that are therapeutically useful in human conditions such as cardiovascular disease inflammatory arthritis, cancer and neurological disorders. Others have been identified as being useful as pesticides and herbicides. It is expected that the invention can be applied to the production of a wide range of secondary metabolites. In particular, the invention can be applied to microbial production of the group of compounds known as taxanes, of which the compound taxol is representative (U.S. Patent Nos. 5,861,302 and 5,322,779).

In another embodiment, the present invention provides improved methods for the production of secondary metabolites by *Streptomyces roseosporus*, especially the A-21978C complex of antibiotics, and even more particularly the n-decanoyl derivative of the A-21978C "peptide core" that has been named daptomycin..

The invention also provides a method for improving the production level of a secondary metabolite of *Streptomyces roseosporus*, comprising culturing *Streptomyces roseosporus* in a nutrient medium, in which *Streptomyces roseosporus* synthesizes an amount of at least one secondary metabolite, and wherein the nutrient medium comprises an amount of at least one organic additive sufficient to increase the production level of the secondary metabolite relative to that in the absence of at least one organic additive.

In another embodiment, the invention provides a method for improving the production level of a secondary metabolite of *Streptomyces roseosporus*, comprising culturing *Streptomyces roseosporus* in a nutrient medium, in which *Streptomyces roseosporus* synthesizes an amount of the secondary metabolite daptomycin, and wherein the nutrient medium comprises an amount of at least one organic additive sufficient to increase the production level of daptomycin relative to that in the absence of the at least one organic additive.

In a further embodiment, the present invention provides a method for enhancing the component profile of secondary metabolites produced by *Streptomyces*

roseosporus, comprising culturing *Streptomyces roseosporus* in a nutrient medium, in which *Streptomyces roseosporus* elaborates a component profile of secondary metabolites, wherein the nutrient medium comprises an amount of at least one organic additive sufficient to enhance the component profile of secondary metabolites produced by *Streptomyces roseosporus* relative to that in the absence of the at least one organic additive.

In a still further embodiment, the present invention provides a method for enhancing the component profile of secondary metabolites produced by *Streptomyces roseosporus*, comprising culturing *Streptomyces roseosporus* in a nutrient medium, in which *Streptomyces roseosporus* elaborates components of the A-21978C complex of secondary metabolites, wherein the nutrient medium comprises an amount of at least one organic additive sufficient to enhance the component profile of A-21978C complex of secondary metabolites produced by *Streptomyces roseosporus* relative to that in the absence of the at least one organic additive.

In a further embodiment, the invention provides a method for identifying a novel secondary metabolite, comprising culturing *Streptomyces roseosporus* in a nutrient medium, wherein the nutrient medium comprises an amount of at least one organic additive sufficient to enhance the component profile of secondary metabolites produced by *Streptomyces roseosporus*, and in which, at least on novel secondary metabolite is identified in the component profile of secondary metabolites produced by *Streptomyces roseosporus*, relative to that in the absence of the at least one organic additive.

The invention and its use are illustrated, but not limited, by the following examples.

6. EXAMPLES

Methodology used in the Examples 1-12

The effects of the organic additives, DMSO, ethanol and dimethyl sulfone, on the production of secondary metabolites in *Streptomyces* were demonstrated as described below in Examples 1-12.

Spore suspensions of the various species of *Streptomyces* (~ 1-2 x 10^8 cfu/ml) used in the present invention were obtained as follows. The bacteria were initially grown on ISP (Difco) agar plates until sporulation. The plate surface was then washed with sterile water to harvest the spores. The spore solution was mixed with an equal volume of 50% glycerol and stored as frozen stocks. The frozen spore stocks, which were titered to establish the viable count, were used as inoculum in the following examples.

The general culturing conditions for the *Streptomyces* species were as follows. Culture medium was first inoculated with the spore suspension and mixed thoroughly. Ten ml of the inoculated culture medium were added to 50 ml test tubes and the cells were grown aerobically at 30°C in the absence or presence of either DMSO, ethanol or dimethyl sulfone.

Analysis of the secondary metabolites produced in the cultures was estimated as follows. At various times during the fermentation, generally after five to seven days, 1 ml of the spent medium with cells was transferred to a 2 ml microfuge tube for extraction. An equal amount of organic extraction solvent was added to the microfuge tube and the tube was horizontally shaken for 30 min. The microfuge tube was centrifuged at 14,000 rpm for 5 minutes at which time 0.75 ml of the organic fraction was transferred to a new 2 ml microfuge tube, dried in an evacuated centrifuge with medium heat, approximately 40°C, then analyzed using a Beckman System Gold HPLC with a reverse C18 column.

Cell biomass for each culture of the various *Streptomyces* species was determined as follows. The remaining spent medium with cells was centrifuged at 4,000 rpm for 10 minutes. The supernatant was discarded and the remaining pellet was resuspended in 40 ml of distilled water. The cell suspension was centrifuged again. The cells were resuspended in distilled water and harvested by filtering the cell suspension through Whatman #1 filter paper. The filter paper with cells was then baked in an oven at 80°C overnight and the dry biomass weighed.

6.1 Example I: *Streptomyces glaucescens*

Cells of the wild type strain of *Streptomyces glaucescens* were grown in Tryptic Soy Broth (TSB) of the following composition:

Bacto tryptone	17 g/L
Bacto soytone	13.0 g/L
Bacto dextrose	2.5 g/L
NaCl	5 g/L
K_2HPO_4	2.5 g/L
Water	to 1 L

Culturing and extraction procedures were as outlined above except that 10 µl of 1 M KH_2PO_4 was added to each 1 ml of culture sample prior to extraction with the solvent ethyl acetate (HPLC grade). The effect of 1, 3 and 5 % (v/v) DMSO on the production of tetracenomycin was analyzed using HPLC. Analytical work was done using a Beckman System Gold HPLC with a reverse C_{18} column (4.5 x 150 mm.) using a gradient of H_2O and CH_3CN from 10:90 to 80:20 within 20 minutes. Authentic standards of tetracenomycin were also chromatographed for comparison.

Figure 1 shows that tetracenomycin production was highest when 3 % v/v DMSO was added. Tetracenomycin production was increased 4.6-fold as compared to control cultures, where no DMSO was added.

6.2 Example II: *Streptomyces venezuelae*

Cells of *Streptomyces venezuelae* strain ATCC10712 were grown in soy flour medium of the following composition:

Glucose	20 g/L
Soybean meal	20 g/L
Yeast extract	1 g/L
$CaCO_3$	2 g/L
Water	to 1 L

Cells were cultured as outlined above and the 1 ml culture samples were extracted with an equal volume of the solvent ethyl acetate (HPLC grade). The effect of 1, 3 and 5 % (v/v) DMSO on the production of chloramphenicol was analyzed with HPLC using a zorbax SB C_{18} rapid column (4.5 x 15 mm.) using a gradient of H_2O and CH_3CN form 80:20 to 0:100 within 5 minutes (1ml/min). Authentic standards of chloramphenicol were also chromatographed for comparison.

Figure 2 shows that chloramphenicol production was highest when 3 % v/v DMSO was added and production started to decline at 5 % v/v DMSO.

6.3 Example III: *Streptomyces venezuelae*

Cells of the wild type strain of *Streptomyces venezuelae* were grown in GNY medium of the following composition:

Glycerol	20 ml/L
Nutrient broth	8 g/L
Yeast extract	3 g/L
K_2HPO_4	5 g/L
Water	to 1 L

Cells were cultured as outlined above and the 1 ml culture samples were extracted with an equal volume of the solvent ethyl acetate (HPLC grade). The effect of 1, 3 and 5 % (v/v) DMSO on the production of chloramphenicol was analyzed with HPLC using the same method outlined above. Authentic standards of chloramphenicol were also chromatographed for comparison.

Figure 3 shows that chloramphenicol production continued to rise even at 5 % v/v DMSO. At that concentration, chloramphenicol production had increased about 10-fold.

6.4 Example IV: *Streptomyces azureus*

Cells of *Streptomyces azureus* strain ATCC14921 were grown in R5 medium of the following composition:

Sucrose	103 g/L
K_2SO_4	0.25 g/L
$MgCl_2 \cdot 6H_2O$	10.12 g/L
Glucose	10 g/L
Casamino acids	40.1 g/L
Yeast extract	5 g/L
TES	5.73 g/L
KH_2PO_4 (0.5%)	10 ml
$CaCl_2.2H_2O$ (5M)	4 ml
L-proline (20%)	15 ml
NaOH (1N)	7 ml

$ZnCl_2$	80 µg/L
$FeCl_3 \cdot 6H_2O$	400 µg/L
$CuCl_2 \cdot 2H_2O$	20 µg/L
$MnCl_2 \cdot 4H_2O$	20 µg/L
$Na_2B_4O_7 \cdot 10H_2O$	20 µg/L
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	20 µg/L
Water	to 1 L

Cells were cultured as outlined above and the 1 ml culture samples were extracted with an equal volume of the solvent chloroform (HPLC grade). The effect of 1, 3 and 5 % (v/v) DMSO on the production of thiostrepton was analyzed with HPLC using a C_8 reverse phase column (Beckman, ultrasphere Octyl, 4.5 x 150 mm) with an isocratic mobile phase of H_2O-THF-CH_3OH (2:2:1) and monitored at 245 nm. Authentic standards of thiostrepton were also chromatographed for comparison.

Figure 4 shows that at 1, 3 and 5 % v/v DMSO the production of thiostrepton almost doubled.

6.5 Example V: Recombinant *Streptomyces lividans* clone S10H10

Clone S10H10, contained in a *Streptomyces lividans* host, is a shuttle vector containing a DNA insert derived from a genomic DNA library, which was made from DNA fragments of approximately 13 Kb from a mixture of previously unknown soil isolates of *Streptomyces*. This clone was examined to ascertain the effect of organic additives on the component profile of secondary metabolites produced by a host *Streptomyces* containing exogenous genetic material.

Clone S10H10 was generated using the shuttle vector plasmid pWHM601. Plasmid pWHM601 was digested with BamHI followed by dephosphorylation with shrimp alkaline phosphotase using standard protocols. Insert DNA with a fragment size of 13 Kb was prepared from partially Sau3AI-digested genomic DNA mixture of ten unknown soil *Streptomyces* isolates 73B, 77D, 830, 85D, 87I, 88A, 91E, 93A, 115C and 118B. The 13 Kb fragments were then ligated to the dephosphoylated plasmid at a ratio of 1:3 using T4 DNA ligase, and directly transformed into the host *Streptomyces lividans* TK23 by protoplast transformation. The transformed protoplasts were spread on R5 agar plates and,

after a 24 hour outgrowth period, were overlaid with a concentrated solution of apramycin, yielding a final concentration of 50 µg apramycin/ml.

Cells of clone S10H10 were cultured in R5 medium containing 25 µg/ml apramycin in the presence and absence of 3% DMSO. One ml samples were extracted using an equal volume of ethyl acetate and subjected to HPLC analysis.

Figure 5b shows the HPLC profile of the low molecular weight metabolites produced by clone S10H10 in the absence of DMSO. Figure 5a shows the HPLC profile when 3% DMSO was added to the culture medium. A comparison of the two profiles shows that DMSO is effective in enhancing the component profile of secondary metabolites of clone S10H10.

6.6 Example VI: Recombinant *Streptomyces lividans* Clone 446-S3-81D3

Clone 446-S3-81D3 is a recombinant consisting of a cosmid vector containing a DNA fragment derived from an environmental DNA sample. The clone was generated using the shuttle cosmid vector pOJ446. The vector was linearized by digestion with HpaI and dephosphorylated with alkaline phosphatase using standard protocols. The linearized vector was further digested with BamHI. Insert DNA with a fractionation size ranging from 23 to 40 Kb was prepared by partial Sau3AI digestion of DNA directly extracted from forestry soil. A cosmid library was generated by ligating the vector to the insert DNA at a ratio of 1:1 using T4 DNA ligase, and packaging *in vitro* using a Gigapack III Gold Packaging Extract kit. The library was first amplified using *Escherichia coli* XL 1 Blue MR as a primary host. Cosmids with insert DNA were then extracted and transferred to the expression host *Streptomyces lividans* TK23 by protoplast transformation. Transformants were selected by growing them on R5 agar plates containing 25 µg apramycin/ml.

Cells of clone 446-S3-8 1 D3 were cultured in R5 medium containing 25 µg apramycin/ml in the presence and absence of various concentrations DMSO. One ml samples were extracted using an equal volume of ethyl acetate and subjected to HPLC analysis.

Figure 6a shows the HPLC profile of the low molecular weight metabolites produced by clone 446-S3-8 1 D3 in the absence of DMSO. Figure 6b shows the HPLC profile when 2% DMSO was added to the culture medium. A comparison of the two profiles shows that DMSO effectively increased the production of many of the secondary metabolites that are produced when the 446-S3-8 1 D3 cells are grown in the absence of

DMSO. The component profile obtained demonstrates that the concentration of some metabolites has increased by as much as six-fold.

6.7 Example VII: Recombinant *Streptomyces lividans* Clone 446-53-102H4

Clone 446-53-102H4 was generated as described in Example VI. Cells of clone 446-53-102H4 were cultured in R5 medium containing 25 µg/ml apramycin in the presence and absence of 2% DMSO. One ml samples were extracted using an equal volume of ethyl acetate and subjected to HPLC analysis.

Figure 7a shows the HPLC profile of the low molecular weight metabolites produced by clone 446-53-102H4 in the absence of DMSO. Figure 7b shows the HPLC profile when 2% DMSO was added to the culture medium. A comparison of the two profiles shows that DMSO effectively increased the production of many of the secondary metabolites that are produced when the 446-S3-1 02H4 cells are grown in the absence of DMSO. The data obtained indicate that the concentration of some metabolites has increased more than ten-fold when the cells are grown in the presence of DMSO.

6.8 Example VIII: *Streptomyces glaucescens*

Cells of the wild type strain of *Streptomyces glaucescens* were grown in Tryptic Soy Broth (TSB) which composition is recited in Example I. Culturing and extraction procedures were as outlined above except that 10 µl of 1 M KH_2PO_4 was added to each 1 ml of culture sample prior to extraction with the solvent ethyl acetate (HPLC grade). The effect of 1, 3 and 5 % (v/v) ethanol on the production of tetracenomycin was analyzed using HPLC. Authentic standards of tetracenomycin were also chromatographed for comparison.

Figure 8 shows that tetracenomycin production was highest when 3 % v/v ethanol was added. However, there was only a 1.5 fold increase in tetracenomycin (TCMC) production as compared to the almost five-fold increase in tetracenomycin observed when 3 % v/v DMSO was added to cultures of *Streptomyces glaucescens*.

6.9 Example IX: *Streptomyces glaucescens*

Cells of the wild type strain of *Streptomyces glaucescens* were grown in R5 medium, the composition of which is recited in Example IV. Culturing and extraction procedures were as outlined above except that 10 µl of 1 M KH_2PO_4 was added to each 1 ml of culture

sample prior to extraction with the solvent ethyl acetate (HPLC grade). The effect of 1, 3 and 5 % (v/v) ethanol on the production of tetracenomycin was analyzed using HPLC. Authentic standards of tetracenomycin were also chromatographed for comparison.

Figure 9 shows that tetracenomycin production was highest when 3% v/v ethanol was added, resulting in a 1.6-fold increase in the production of tetracenomycin.

6.10 Example X: *Streptomyces venezuelae*

Streptomyces venezuelae cells were cultured in GNY medium with 1, 3, and 5% dimethyl sulfone (w/v) and analyzed as described in Example III. Figure 10 shows that production of chloramphenicol was almost doubled at 1% dimethyl sulfone.

6.11 Example XI: *Taxomyces andreanae*

Cells of *Taxomyces andreanae* are grown as described in U.S. Patent Nos. 5,322,779 and 5,861,302, which are incorporated herein in their entirety, in Taxol Microbial Culture Medium of the following composition:

Glucose	1 g/l
Fructose	3 g/l
Sucrose	6 g/l
$KHPO_4$-KH_2PO_4	1 ml of 1M pH 6.8
$MgSO_4$	0.36 g/l
$Ca(NO_3)_2H_2O$	0.65 g/l
Yeast extract	0.5 g/l
$Ca(NO_3)_2$	1.0 mg/l
$ZnSO_4$	2.5 g/l
$MnCl_2$	0.5 g/l
FeC_2	2.0 g/l
Leucine	0.1 mM
Phenylalanine	0.01 mM
NaAc	1 mM
Water	up to 1 l

Incubation of the fungus is carried out at 25°C, without agitation, under still conditions for 3 weeks. At the end of the incubation period, the spent medium is decanted and the mycelium is thoroughly ground at maximum speed in a Sorvall Omnimixer for 30 seconds. An equal volume of chloroform-methanol solution (10:1 v/v) is added to the medium and ground mycelium, and then the chloroform layer is removed. A sample of the chloroform layer is dried and subjected to chromatography on silica gel plates. Authentic standards of taxol are also chromatographed for comparison.

To increase the amount of taxol produced, at least one organic additive, preferably DMSO, ethanol or dimethyl sulfone, is added to the culture medium. The concentration of the organic additives in the culture medium is in the range of about 0.1 to about 10% v/v.

6.12 Example XII: *Streptomyces roseosporus*

Cells of *Streptomyces roseosporus* are grown in a soy flour medium of the following composition:

Glucose	20 g/L
Soybean meal	20 g/L
Yeast extract	1 g/L
Molasses	5 g/L
$CaCO_3$	2 g/L
Water	to 1 L

Spore suspensions of the *Streptomyces roseosporus* (~ 1-2 x 10^8 cfu/ml) are obtained as follows. The bacteria are initially grown on ISP (Difco) agar plates until sporulation. The plate surface is then washed with sterile water to harvest the spores. The spore solution is mixed with an equal volume of 50% glycerol and stored as frozen stocks. The frozen spore stocks, which are titered to establish the viable count, are used as inoculum in the following example.

The frozen spore stocks are used to inoculate the fermentation medium directly. Alternatively the spore stock are used to inoculate 10 ml. of a seed medium, *e.g.* Tryptic Soy Broth (TSB) that is incubated with shaking for 24 to 48 hrs. at 30° C.

The culturing conditions for the *Streptomyces roseosporus* species are as follows. Culture medium, generally 25 ml. of soy flour-molasses medium in a 250 ml.

baffled flask is inoculated with the spore suspension or with a seed culture (generally 1 ml) and mixed thoroughly. The cells are grown aerobically at 30°C in the absence or presence of either DMSO, for seven days.

Analysis of the secondary metabolites produced in the cultures is estimated as follows. At various times during the fermentation, generally after five to seven days, aliquots of the fermentation are removed and centrifuged, for example, at 5000 rpm in a bench-top centrifuge, using sufficient force to pellet the cells. Samples of the supernatant are analyzed directly by HPLC or an ethyl acetate extract of the supernatant is prepared and analyzed by HPLC. The cell pellet is extracted with ethyl acetate and the extract analyzed by HPLC. Alternatively, the spent medium, containing the *Streptomyces roseosporus* cells may be extracted directly as follows. 1 ml of the spent medium with cells is transferred to a 2 ml microfuge tube for extraction. An equal amount of organic extraction solvent, ethyl acetate is added to the microfuge tube and the tube is horizontally shaken for 30 min. The microfuge tube is centrifuged at 14,000 rpm for 5 minutes at which time 0.75 ml of the organic fraction is transferred to a new 2 ml microfuge tube, dried in an evacuated centrifuge with medium heat, approximately 40°C, and then analyzed using a Beckman System Gold HPLC with a reverse C18 column.

Cell biomass for each culture of *Streptomyces roseosporus* is determined as follows. The remaining spent medium with cells is centrifuged at 4,000 rpm for 10 minutes. The supernatant is discarded and the remaining pellet is resuspended in 40 ml of distilled water. The cell suspension is centrifuged again. The cells are resuspended in distilled water and harvested by filtering the cell suspension through Whatman #1 filter paper. The filter paper with cells is then baked in an oven at 80°C overnight and the dry biomass weighed.

Cells are cultured as outlined above and a 1 ml culture samples is extracted with an equal volume of the solvent ethyl acetate (HPLC grade). The effect of 1, 3 and 5 % (v/v) DMSO on the production of daptomycin is analyzed by HPLC using a zorbax SB C_{18} rapid column (4.5 x 15 mm.) using a gradient of H_2O and CH_3CN from 80:20 to 0:100 within 5 minutes (1ml/min). Cells are also grown in the absence of DMSO and analyzed as above, for comparison of the effects of DMSO on daptomycin production levels, component profile, and for detection of new components that may be novel secondary metabolites elaborated by *Streptomyces roseosporus*. Authentic standards of daptomycin are also chromatographed for comparison.

The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and

functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are incorporated by reference herein in their entireties for all purposes.

What is claimed is:

1. A method for improving a production level of a secondary metabolite, comprising culturing at least one organism in a nutrient medium, wherein the at least one organism synthesizes an amount of at least one secondary metabolite, and wherein the nutrient medium comprises an amount of at least one organic additive sufficient to increase the production level of the secondary metabolite relative to that in the absence of the at least one organic additive.

2. A method for enhancing a component profile of secondary metabolites, comprising culturing at least one organism in a nutrient medium, wherein the at least one organism elaborates a component profile of secondary metabolites, and wherein the nutrient medium comprises an amount of at least one organic additive sufficient to enhance the component profile of secondary metabolites relative to that in the absence of the at least one organic additive.

3. A method for identifying a novel secondary metabolite, comprising culturing at least one organism in a nutrient medium, wherein the nutrient medium comprises an amount of at least one organic additive sufficient to enhance a component profile of secondary metabolites, wherein the novel secondary metabolite is identified in the component profile relative to that in the absence of the at least one organic additive.

4. The method of claim 1, 2, or 3, wherein the organic additive is present at a concentration of between about 0.1% and about 10 % v/v.

5. The method of claim 1, 2, or 3, wherein the organic additive is present at a concentration of between about 0.5% and about 7.5 % v/v.

6. The method of claim 1, 2, or 3, wherein the organic additive is present at a concentration of between about 1 % and about 5% v/v.

7. The method of claim 1, 2, or 3, wherein the organic additive is an organic molecule having a molecular weight of less than about 250, wherein the organic molecule comprises at least one heteroatom selected from the group consisting of sulfur, oxygen, and combinations thereof.

8. The method of claim 7, wherein the organic additive is a lower alcohol selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol, n-butanol, iso-butanol, t-butanol, amyl alcohol, iso-amyl alcohol, and combinations thereof.

9. The method of claim 1,2, or 3, wherein the at least one organic additive is selected from the group consisting of dimethyl sulfoxide, ethanol, dimethyl sulfone and combinations thereof.

10. The method of claim 1, 2, or 3, wherein the organic additive is dimethyl sulfoxide.

11. The method of claim 1, 2, or 3, wherein the organic additive is ethanol.

12. The method of claim 1, 2, or 3, wherein the organic additive is dimethyl sulfone.

13. The method of claim 1, 2, or 3, wherein the at least one organism is cultured in a process selected from the group consisting of batch fermentations, fed-batch fermentations, continuous fermentations, co-synthetic fermentations, mutasynthetic fermentations, immobilized-cell fermentations, fermentations on semi-solid media, and combinations thereof.

14. The method of claim 1, 2, or 3, wherein the at least one organism comprises a recombinant organism.

15. The method of claim 1, 2, or 3, wherein the at least one organism comprises a bacterium.

16. The method of claim 15, wherein the bacterium belongs to the family *Actinomyces*.

17. The method of claim 16, wherein the organism belongs to a genus selected from the group comprising *Streptomyces, Actinomadura, Actinoplanes, Actinosporangium, Actinosynnema, Ampullariella, Chainia, Dactylosporangium, Kibdelosporangium, Kitasatosporia, Kitasatoa, Microellobosporia, Micromonospora, Microtetraspora,*

Nocardia, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Streptoalloteichus*, *Streptosporangium*, *Streptoverticillum*, *Thermoactinomyces*, and *Thermomonospora*.

18. The method of claim 17, wherein the organism belongs to the genus *Streptomyces*.

19. The method of claim 17, wherein the organism is selected from the group consisting of *Streptomyces hygroscopicus*, *Streptomyces halstedii*, *Streptomyces erythrae*, *Saccharopolyspora erythrae*, *Streptomyces mediterranei*, *Streptomyces aureofaciens*, *Streptomyces venezuelae*, *Streptomyces spheroides*, *Streptomyces cinnamonensis*, *Streptomyces albus*, *Streptomyces caespitosus*, *Streptomyces avermitilis*, *Streptomyces parvulus*, *Streptomyces antibioticus*, *Streptomyces azureus*, *Streptomyces griseus*, *Streptomyces clavuligeris*, *Streptomyces lavendulae*, *Streptomyces lincolnensis*, *Streptomyces curacoi*, *Streptomyces fradiae*, *Streptomyces rimosus*, *Streptomyces peucetius*, *Streptomyces lividans*, *Streptomyces sp.* (ATCC accession number 55656), and *Streptomyces roseosporus*.

20. The method of claim of claim 15, wherein the organism is selected from the group consisting of *Pseudomonas*, *Bacillus*, *Myxococcales*, *Rhizobia*, and *Cyanobacteria*.

21. The method of claim 1, 2, or 3, wherein the organism is a fungus.

22. The method of claim 21, wherein the organism belongs to a genus selected from the group comprising *Penicillium*, *Cephalosporium*, *Taxomyces*, *Monascus*, *Heterobasidion*, *Genisculosporium*, *Byssochlamys* and *Aspergillus*.

23. The method of claim 1, 2, or 3, wherein the organism is a lichen.

24. The method of claim 1, 2, or 3, wherein the organism is a plant.

25. The method of claim 24, wherein the plant belongs to a genus selected from the group consisting of: *Catharanthus*, *Perilla*, *Taxus*, *Curcuma*, *Hevea*, *Pinus*, *Eschscholzia*, *Hypericum*, and *Brassica*.

26. The method of claim 1, wherein the at least one metabolite comprises tetracenomycin.

27. The method of claim 1, wherein the at least one metabolite comprises chloramphenicol.

28. The method of claim 1, wherein the at least one metabolite comprises thiostrepton.

29. The method of claim 1, wherein the at least one metabolite comprises a taxane.

30. The method of claim 1, wherein the at least one metabolite comprises taxol.

31. The method of claim 3, wherein the nutrient medium is semi-solid.

32. A medium for improving a production level of a secondary metabolite comprising culturing at least one organism in a nutrient medium, wherein the at least one organism synthesizes an amount of at least one secondary metabolite, and wherein the nutrient medium comprises an amount of at least one organic additive sufficient to improve the production level of the at least one secondary metabolite relative to that in the absence of the at least one organic additive.

33. A medium for enhancing a component profile of secondary metabolites comprising culturing at least one organism in a nutrient medium, wherein the at least one organism elaborates a component profile of secondary metabolites, and wherein the nutrient medium comprises an amount of at least one organic additive sufficient to enhance the component profile of secondary metabolites relative to that in the absence of the at least one organic additive.

34. The method of claim 32 or 33, wherein the organic additive is present at a concentration of between about 0.1% and about 10 % v/v.

35. The method of claim 32 or 33, wherein the organic additive is present at a concentration of between about 0.5% and about 7.5 % v/v.

36. The method of claim 32 or 33, wherein the organic additive is present at a concentration of between about 1% and about 5% v/v.

37. The method of claim 32 or 33, wherein the at least one organic additive is selected from the group consisting of dimethyl sulfoxide, ethanol, dimethyl sulfone and combinations thereof.

38. A method for isolating a novel secondary metabolite comprising :

(a) culturing at least one organism in a nutrient medium, wherein the at least one organism elaborates a first component profile of secondary metabolites;

(b) culturing the at least one organism in the nutrient medium, wherein the nutrient medium further comprises an amount of at least one organic additive sufficient to enhance a component profile of secondary metabolites, wherein the at least one organism elaborates a second component profile of secondary metabolites;

(b) comparing the second component profile to the first component profile, thereby identifying at least one novel component in the second component profile, wherein the novel component comprises at least one compound; and

(d) purifying the at least one compound.

39. A method for detection of secondary metabolites, comprising culturing at least one organism in a nutrient medium, wherein the at least one organism elaborates at least one secondary metabolite, and wherein the nutrient medium comprises at least one organic additive sufficient to provide detection of secondary metabolites relative to that in the absence of the at least one organic additive.

40. The method of claim 1, wherein said organism is *Streptomyces roseosporus*.

41. The method of claim 40, wherein said secondary metabolite is daptomycin.

42. The method of claim 2, wherein said organism is *Streptomyces roseosporus*.

43. The method of claim 42, wherein said component profile of secondary metabolites comprises components of the A-21978C antibiotic complex.

44. The method of claim 43, wherein said component profile of secondary metabolites comprises daptomycin.

45. The method of claim 3, wherein said organism is *Streptomyces roseosporus*.

46. The method of claim 45, wherein said component profile of secondary metabolites comprises daptomycin.

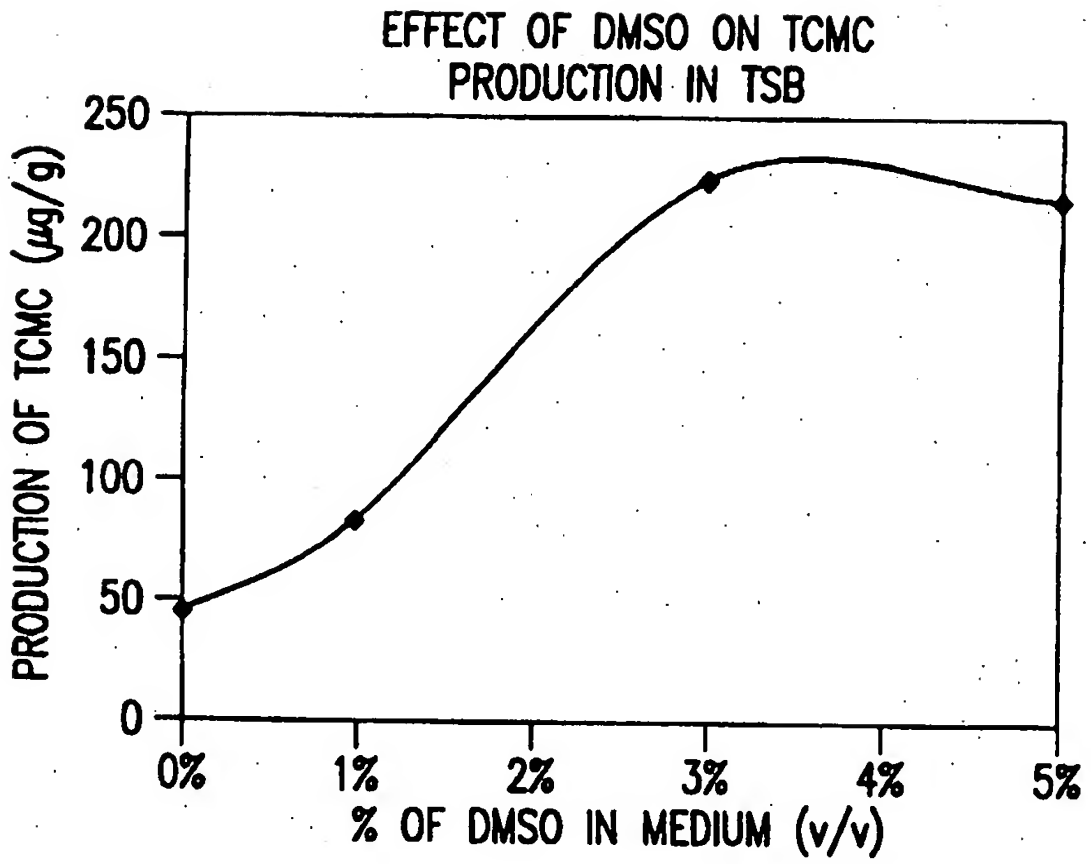
EFFECT OF DMSO ON TCMC PRODUCTION IN TSB
PRODUCTION OF TCMC (μg/g)
250
200
150
100
50
0
0%
1%
2%
3%
4%
5%
% OF DMSO IN MEDIUM (v/v)

FIG.1

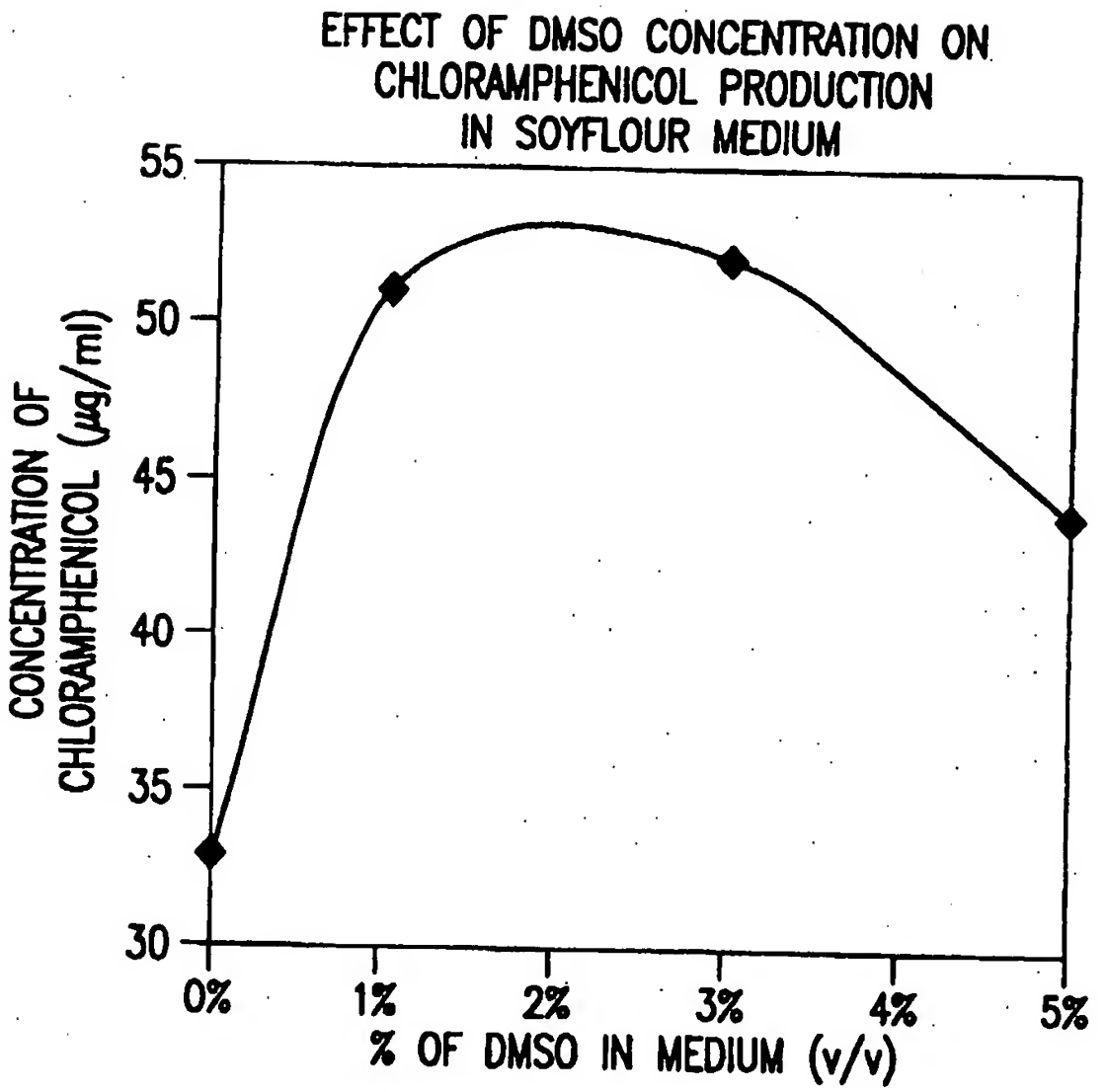
EFFECT OF DMSO CONCENTRATION ON
CHLORAMPHENICOL PRODUCTION
IN SOYFLOUR MEDIUM
CONCENTRATION OF
CHLORAMPHENICOL (µg/ml)
55
50
45
40
35
30
0%
1%
2%
3%
4%
5%
% OF DMSO IN MEDIUM (v/v)

FIG.2

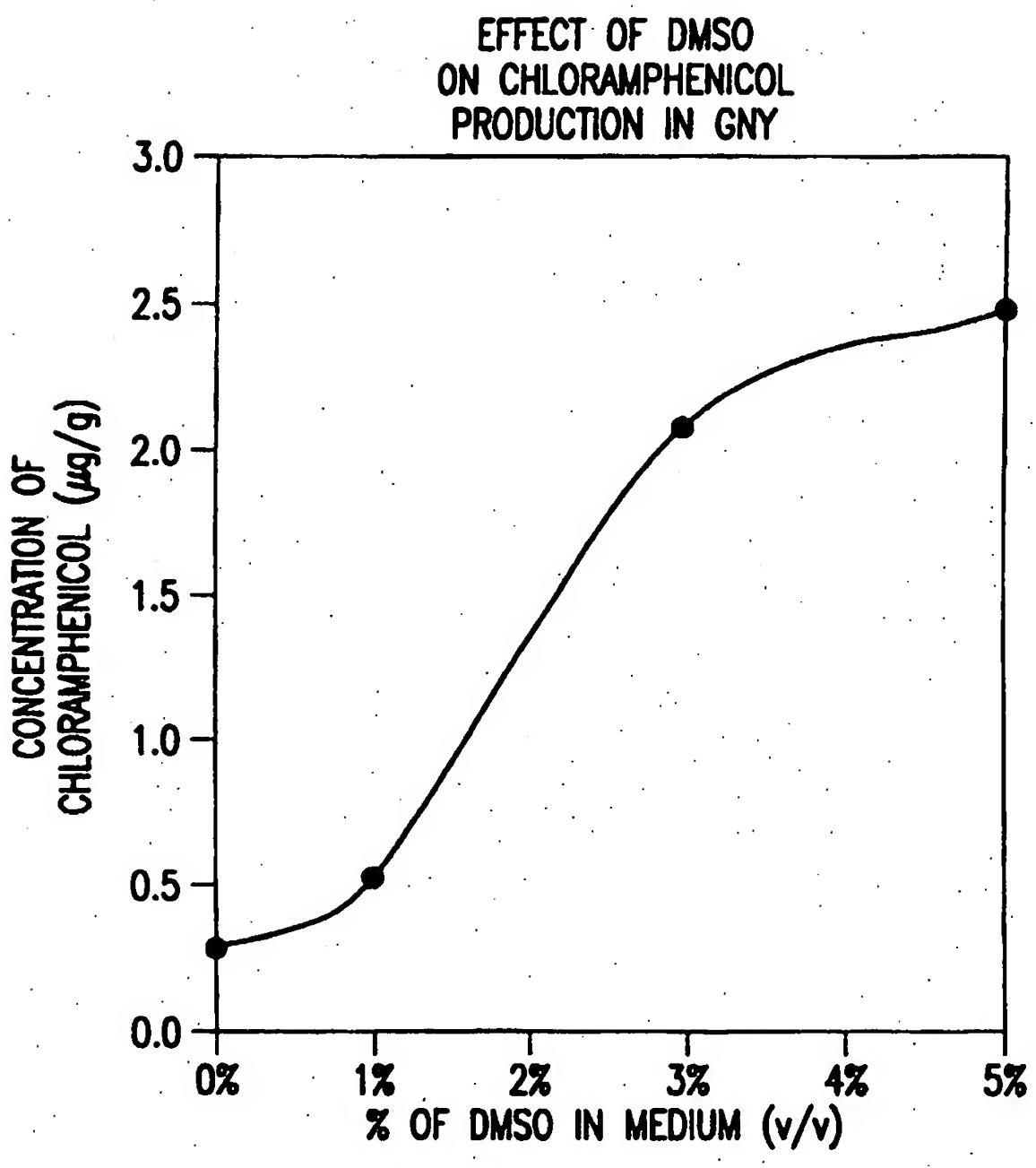
EFFECT OF DMSO
ON CHLORAMPHENICOL
PRODUCTION IN GNY
CONCENTRATION OF
CHLORAMPHENICOL (µg/g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0%
1%
2%
3%
4%
5%
% OF DMSO IN MEDIUM (v/v)

FIG.3

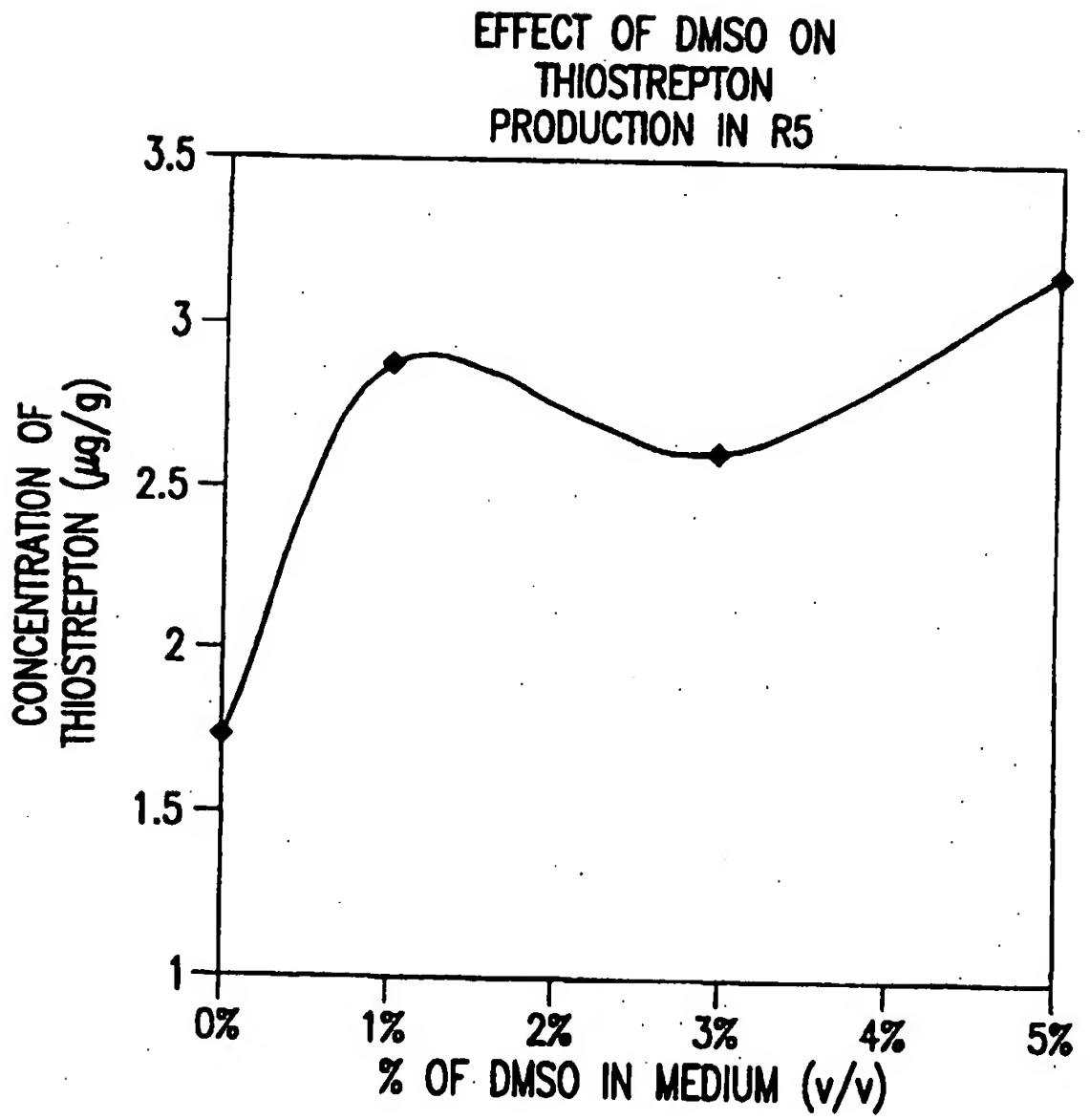
EFFECT OF DMSO ON
THIOSTREPTON
PRODUCTION IN R5
CONCENTRATION OF
THIOSTREPTON (µg/g)
3.5
3
2.5
2
1.5
1
0%
1%
2%
3%
4%
5%
% OF DMSO IN MEDIUM (v/v)

FIG.4

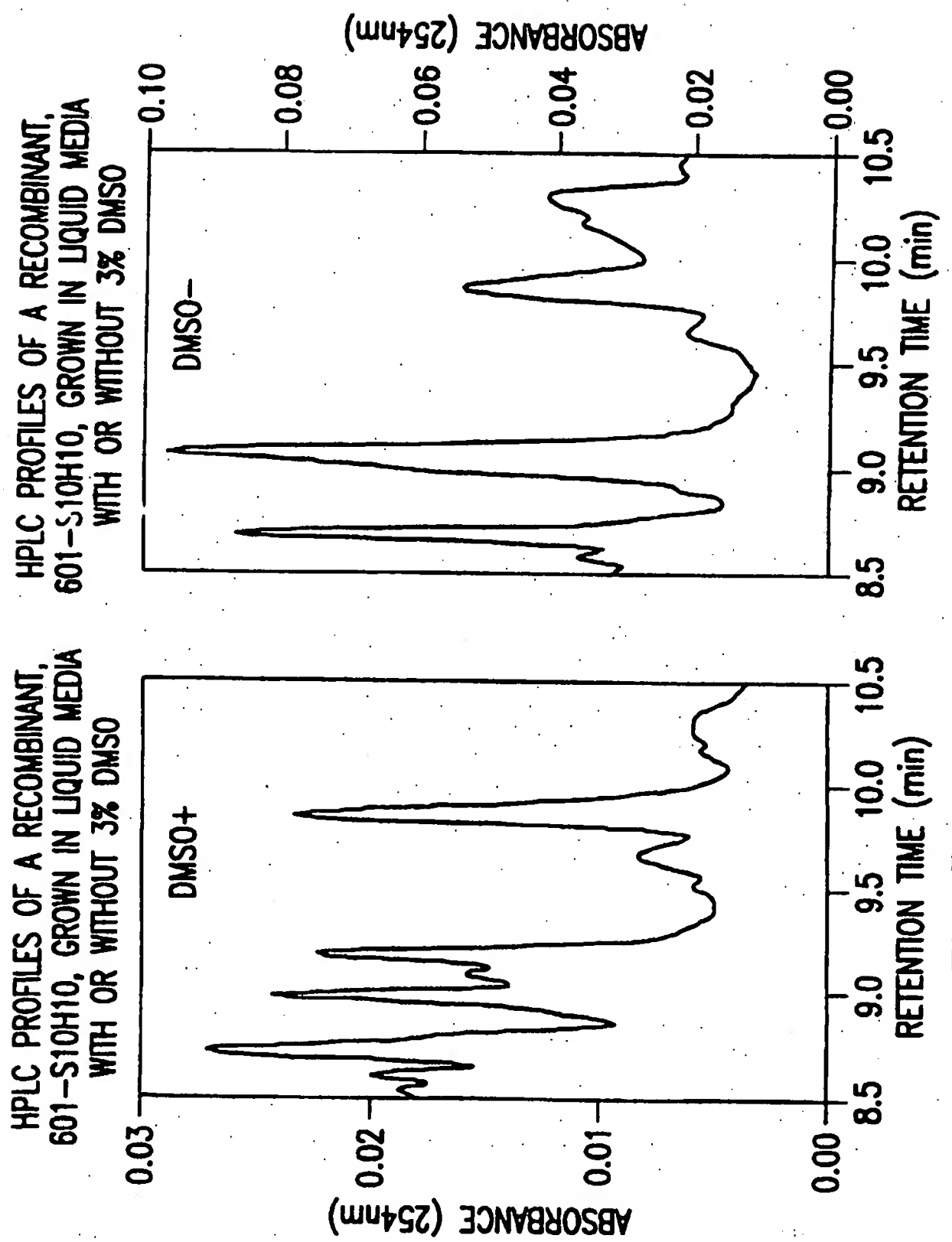
HPLC PROFILES OF A RECOMBINANT,
601-S10H10, GROWN IN LIQUID MEDIA
WITH OR WITHOUT 3% DMSO
DMSO-
ABSORBANCE (254nm)
0.10
0.08
0.06
0.04
0.02
0.00
8.5
9.0
9.5
10.0
10.5
RETENTION TIME (min)

FIG.5B

HPLC PROFILES OF A RECOMBINANT,
601-S10H10, GROWN IN LIQUID MEDIA
WITH OR WITHOUT 3% DMSO
DMSO+
ABSORBANCE (254nm)
0.03
0.02
0.01
0.00
8.5
9.0
9.5
10.0
10.5
RETENTION TIME (min)

FIG.5A

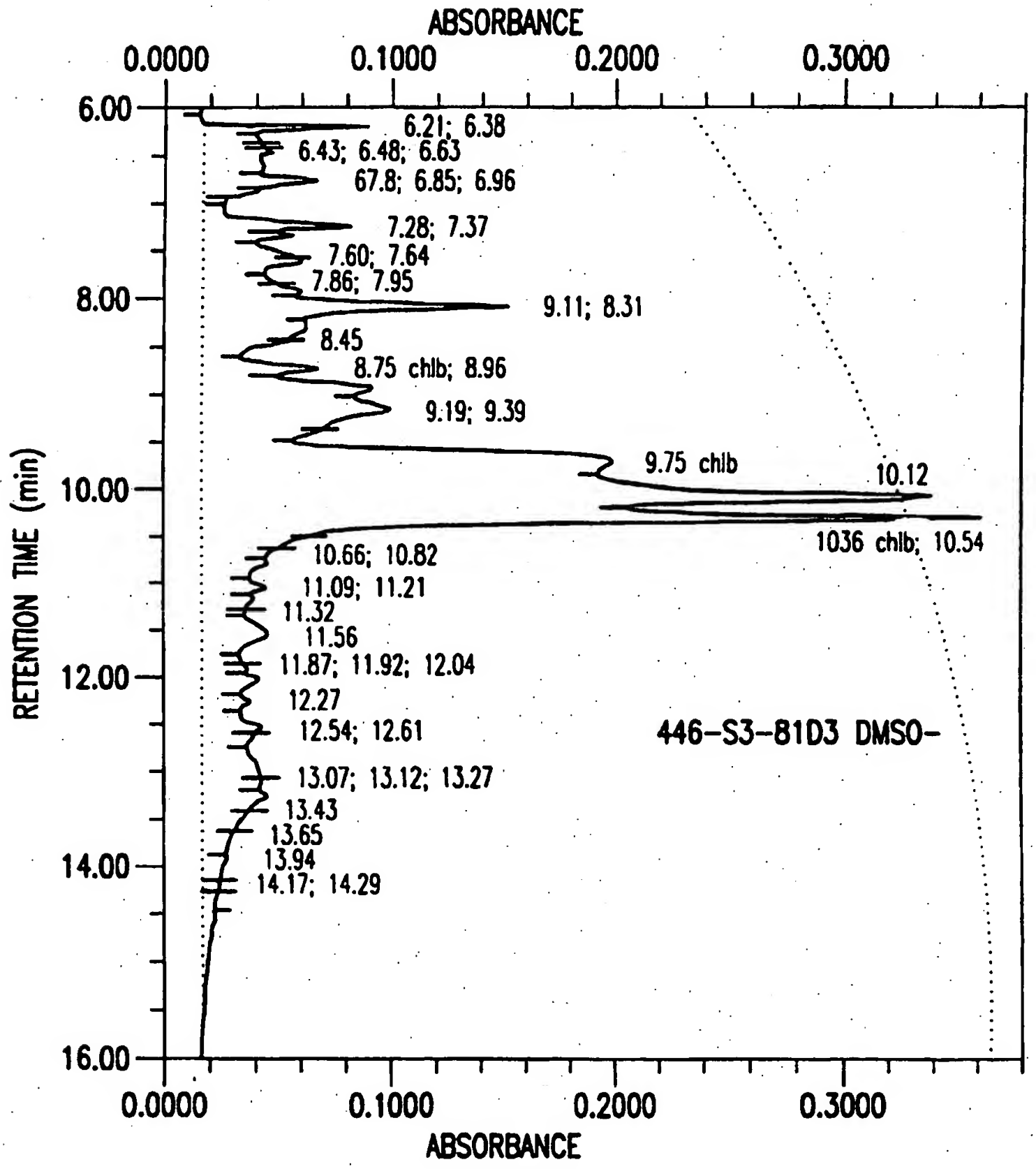
ABSORBANCE
0.0000
0.1000
0.2000
0.3000
RETENTION TIME (min)
6.00
8.00
10.00
12.00
14.00
16.00
6.21; 6.38
6.43; 6.48; 6.63
67.8; 6.85; 6.96
7.28; 7.37
7.60; 7.64
7.86; 7.95
9.11; 8.31
8.45
8.75 chlb; 8.96
9.19; 9.39
9.75 chlb
10.12
1036 chlb; 10.54
10.66; 10.82
11.09; 11.21
11.32
11.56
11.87; 11.92; 12.04
12.27
12.54; 12.61
13.07; 13.12; 13.27
13.43
13.65
13.94
14.17; 14.29
446-S3-81D3 DMSO-
0.0000
0.1000
0.2000
0.3000
ABSORBANCE

FIG. 6A

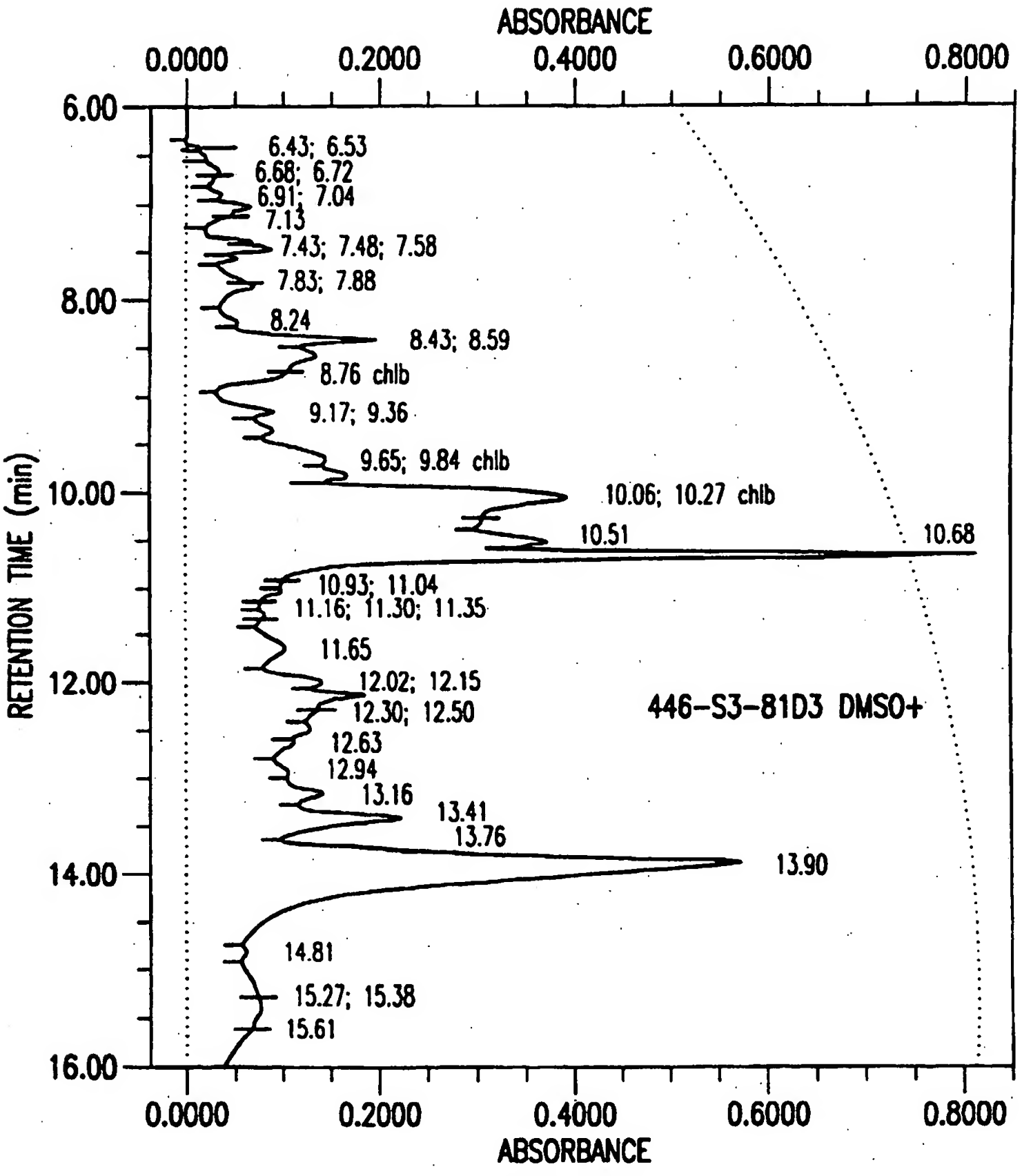
ABSORBANCE
0.0000
0.2000
0.4000
0.6000
0.8000
RETENTION TIME (min)
6.00
8.00
10.00
12.00
14.00
16.00
6.43; 6.53
6.68; 6.72
6.91; 7.04
7.13
7.43; 7.48; 7.58
7.83; 7.88
8.24
8.43; 8.59
8.76 chlb
9.17; 9.36
9.65; 9.84 chlb
10.06; 10.27 chlb
10.51
10.68
10.93; 11.04
11.16; 11.30; 11.35
11.65
12.02; 12.15
12.30; 12.50
12.63
12.94
13.16
13.41
13.76
13.90
14.81
15.27; 15.38
15.61
446-S3-81D3 DMSO+

FIG. 6B

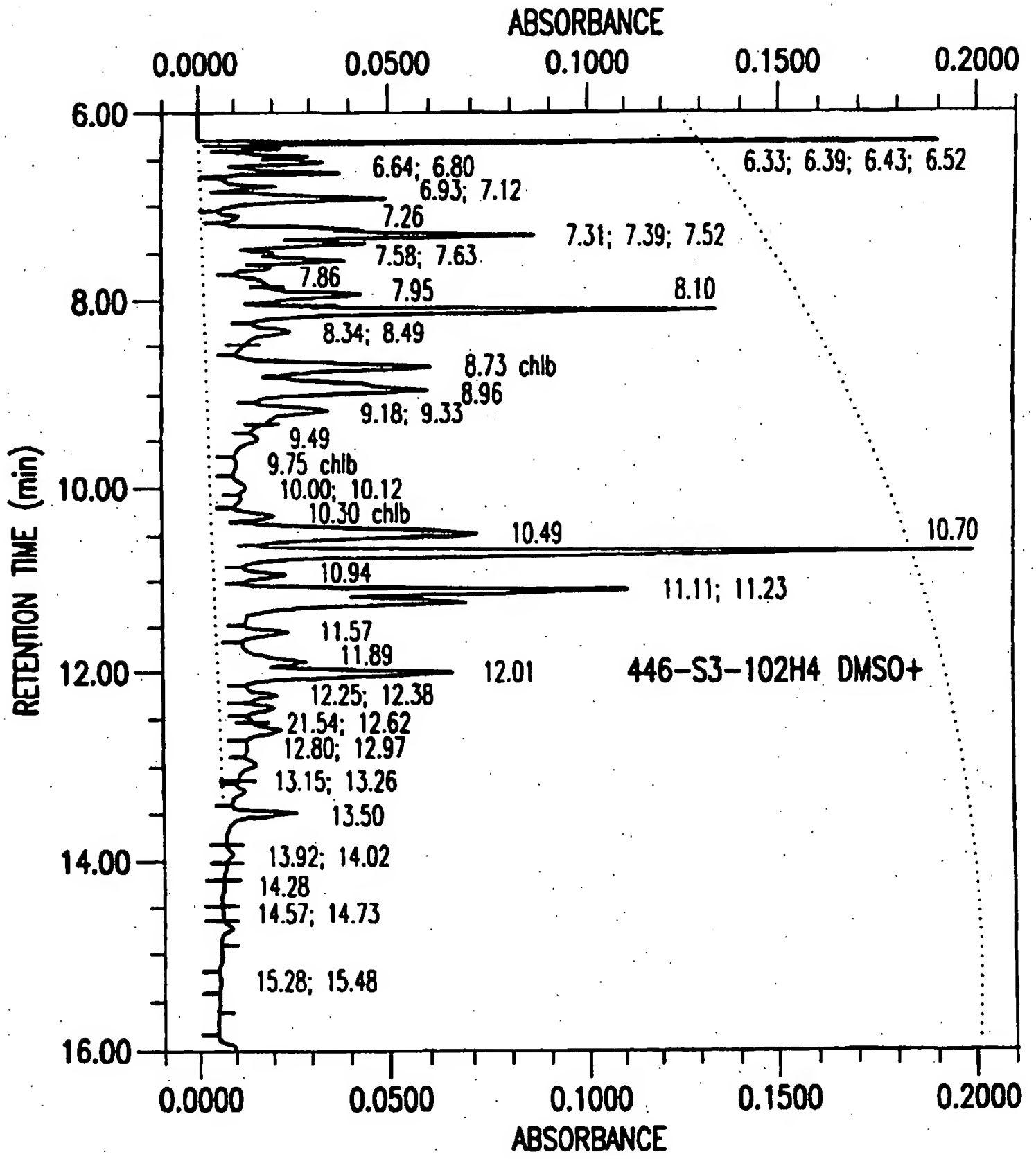
ABSORBANCE
0.0000
0.0500
0.1000
0.1500
0.2000
RETENTION TIME (min)
6.00
8.00
10.00
12.00
14.00
16.00
6.33; 6.39; 6.43; 6.52
6.64; 6.80
6.93; 7.12
7.26
7.31; 7.39; 7.52
7.58; 7.63
7.86
7.95
8.10
8.34; 8.49
8.73 chlb
8.96
9.18; 9.33
9.49
9.75 chlb
10.00; 10.12
10.30 chlb
10.49
10.70
10.94
11.11; 11.23
11.57
11.89
12.01
446-S3-102H4 DMSO+
12.25; 12.38
21.54; 12.62
12.80; 12.97
13.15; 13.26
13.50
13.92; 14.02
14.28
14.57; 14.73
15.28; 15.48

FIG.7A

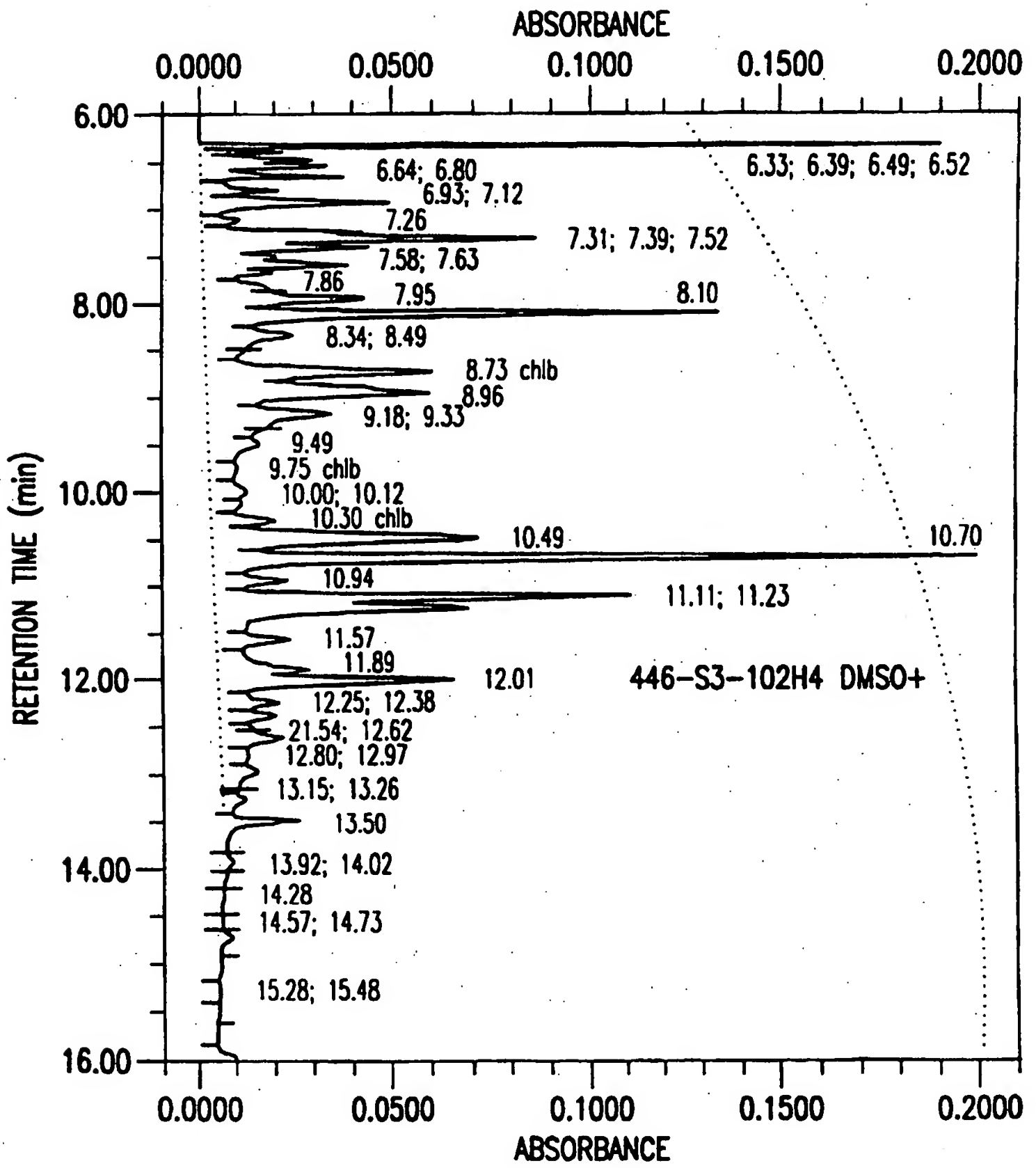
ABSORBANCE
0.0000
0.0500
0.1000
0.1500
0.2000
RETENTION TIME (min)
6.00
8.00
10.00
12.00
14.00
16.00
6.33; 6.39; 6.49; 6.52
6.64; 6.80
6.93; 7.12
7.26
7.31; 7.39; 7.52
7.58; 7.63
7.86
7.95
8.10
8.34; 8.49
8.73 chlb
8.96
9.18; 9.33
9.49
9.75 chlb
10.00; 10.12
10.30 chlb
10.49
10.70
10.94
11.11; 11.23
11.57
11.89
12.01
446-S3-102H4 DMSO+
12.25; 12.38
21.54; 12.62
12.80; 12.97
13.15; 13.26
13.50
13.92; 14.02
14.28
14.57; 14.73
15.28; 15.48

FIG.7B

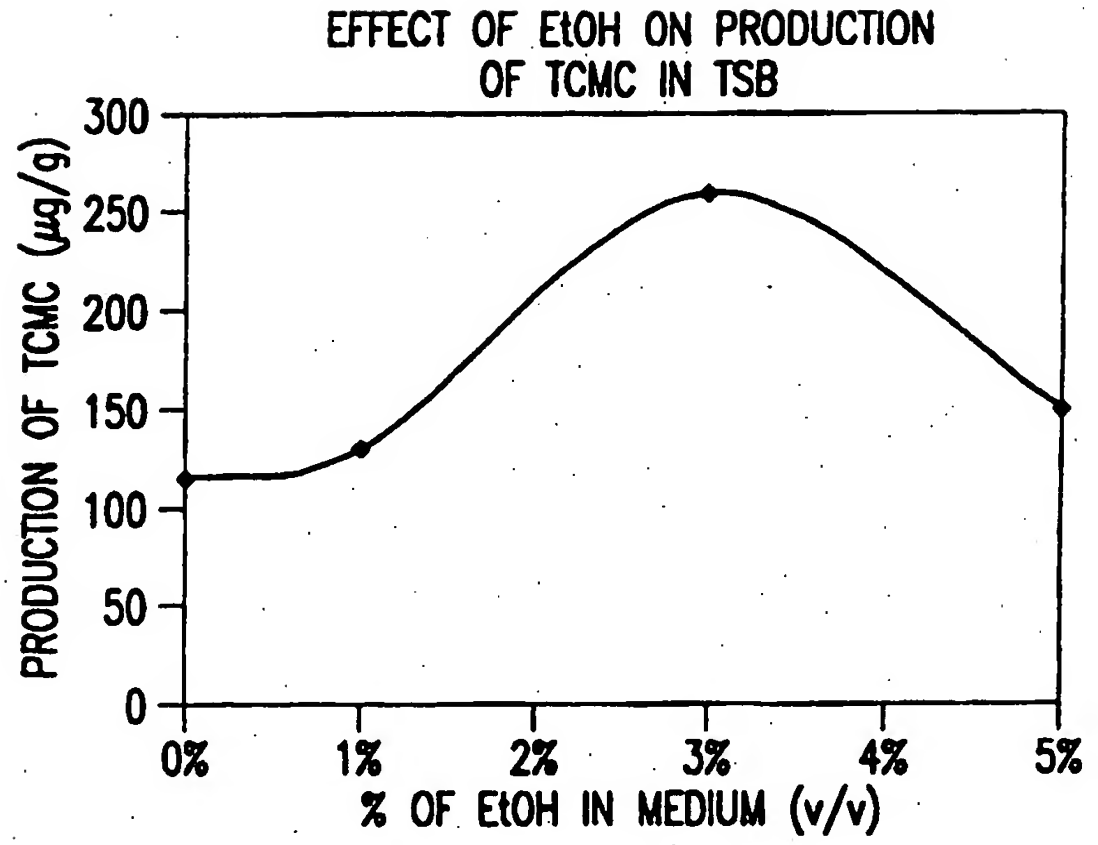
EFFECT OF EtOH ON PRODUCTION
OF TCMC IN TSB
PRODUCTION OF TCMC (µg/g)
300
250
200
150
100
50
0
0%
1%
2%
3%
4%
5%
% OF EtOH IN MEDIUM (v/v)

FIG.8

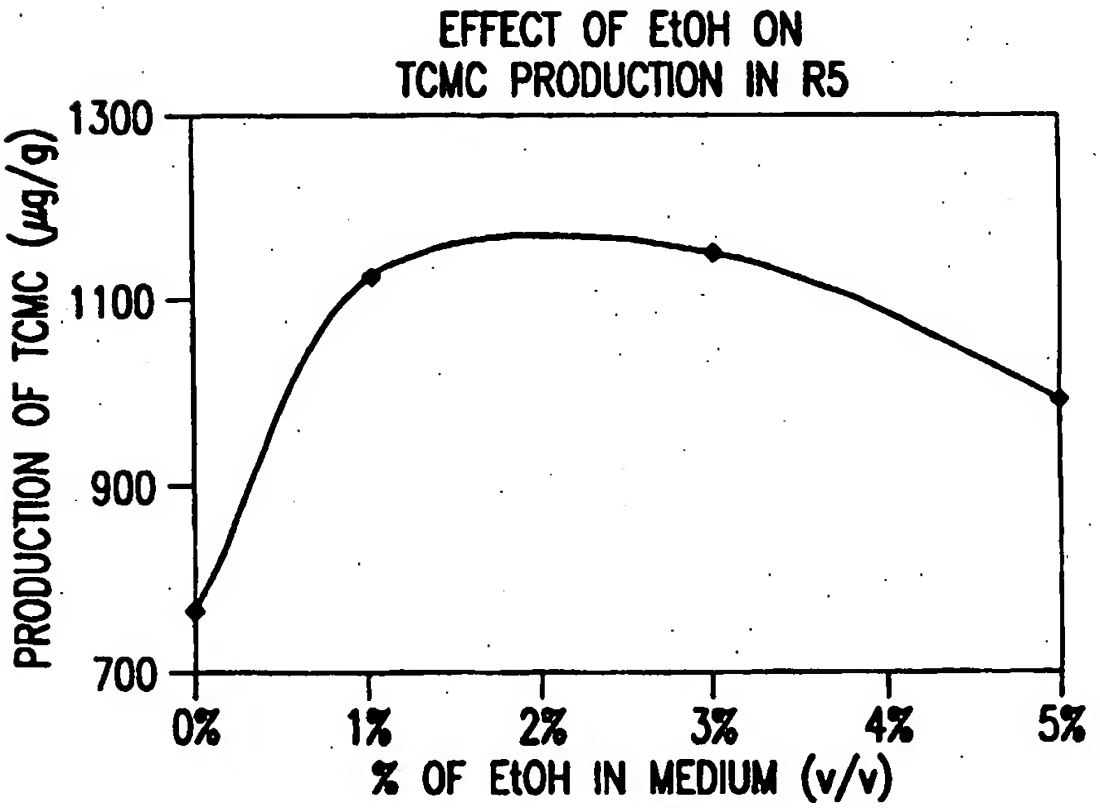
EFFECT OF EtOH ON
TCMC PRODUCTION IN R5
PRODUCTION OF TCMC (µg/g)
1300
1100
900
700
0%
1%
2%
3%
4%
5%
% OF EtOH IN MEDIUM (v/v)

FIG.9

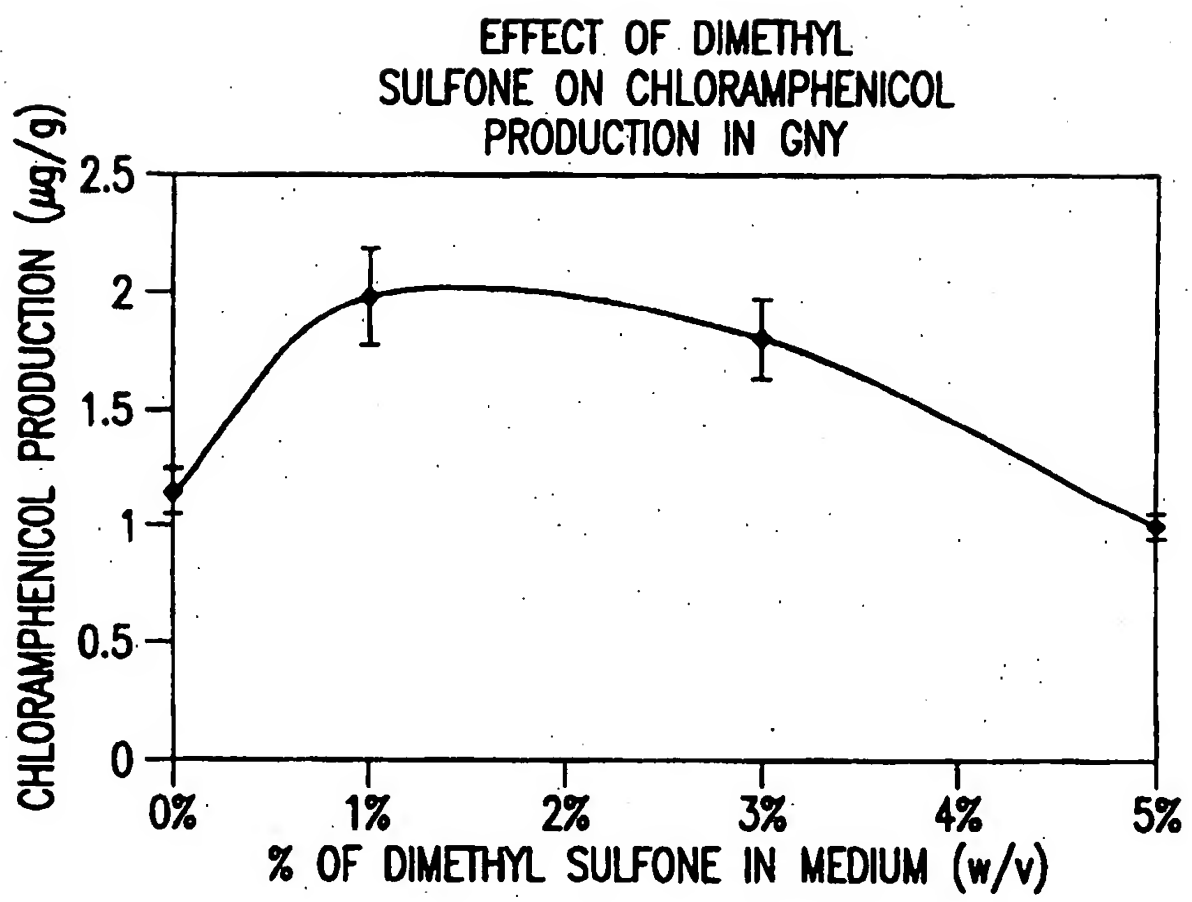
EFFECT OF DIMETHYL SULFONE ON CHLORAMPHENICOL PRODUCTION IN GNY
CHLORAMPHENICOL PRODUCTION (µg/g)
2.5
2
1.5
1
0.5
0
0%
1%
2%
3%
4%
5%
% OF DIMETHYL SULFONE IN MEDIUM (w/v)

FIG.10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/40398

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 1/00, 13/02, 29/00, 17/02, 17/14
US CL : 435/41, 129, 64, 123, 120

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/41, 129, 64, 123, 120

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	DOULL et al. Conditions for the production of jadomycin B by Streptomyces venezuelae ISP5230: effects of heat shock, ethanol treatment and phage infection. J. Industrial Microbiol. 1994, Vol. 13, pages 120-125, especially Abstract.	1, 4-9, 11, 13, 15-19, 27, 32, 34-37 --- 14
X --- Y	TOMBO et al. Diastereoselective Microbial Hydroxylation of Milbemycin Derivatives. Agric. Biol. Chem. 1989, Vol. 53. No. 6, pages 1531-1535, especially Abstract and page 1534 (Table II).	1, 4-11, 13, 15-18, 32, 34-37 --- 14
X --- Y	SABOURIN et al. Biodegradation of Dimethylsilanediol in Soils. Applied and Environmental Microbiol. December 1996, Vol. 62. No. 12, pages 4352-4360, especially Abstract, page 4355 left column, page 4356 Table 4, page 4357, right column, and page 4358 left column.	1, 7-9, 12, 13, 15, 21, 32, 37 --- 14
X --- Y	TOYAMA et al. Three Distinct Quinoprotein Alcohol Dehydrogenases are Expressed when Pseudomonas putida is Grown on Different Alcohols. J. of Bacteriol. May 1995, Vol. 177. No. 9, pages 2442-2450, especially Abstract, page 2443 left column, page 2444, right column, and page 2445 Table 2.	1, 4, 7-9, 11, 13, 15, 20, 32, 34, 37 --- 14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 03 July 2001 (03.07.2001)	Date of mailing of the international search report 07 AUG 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Ponnathapura Achutamurthy Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/40398

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	Database CAPLUS on STN, AN 1981:11311. SQUIBB et al. "Modified method or penicillin fermentation". Jpn. Kokai Tokkyo Koho, 3 pp. JP 55-144897 A2 (Squibb, E.R., and Sons, Inc., USA) 12 November 1980 (12.11.1980), Abstract.	1, 7-9, 11, 13, 21, 22, 32, 37 — 14
X — Y	YAMAMOTO et al. Effects of Culture Conditions on the Growth of Usneaceae Lichen Tissue Cultures. Plant Cell Physiol. 1987, Vol. 28. No. 8, pages 1421-1426, especially Abstract and page 1421.	1, 4-7, 13, 23, 32, 34-36 — 14
X — Y	YU et al. The induction effect of methyl-jasmonate on taxol biosynthesis. Tianran Chanwu Yanjiu Kaifa. 1999, Vol. 11. No. 5, pages 1-7, especially attached database Abstract.	1, 7, 13, 24, 25, 29, 30, 32 — 14
X — Y	UDVARNOKI et al. Biosynthetic Origin of the Oxygen Atoms of Tetracenomycin C. Angew. Chem. Int. Ed. Engl. 1995, Col. 34. No. 5, pages 565-567, especially page 566 left column.	1, 7, 13, 15-18, 26, 32 — 14
X	OGATA et al. Screening of compounds stimulating spore formation and mycelial growth of pock-forming plasmid-carrying strains in Streptomyces azureus. Appl. Microbiol. Biotechnol. 1992, Vol. 37, pages 652-654, especially Abstract.	1, 7, 14-19, 28, 32
X — Y	US 4,885,243 A (HUBER et al.) 05 December 1989 (05.12.1989), column 1, lines 55-60 and column 2, lines 43-45.	1, 7, 13, 15-19, 32, 40, 41 — 14
X, P — Y, P	CHEN et al. Enhanced Production of Microbial Metabolites in the Presence of Dimethyl Sulfoxide. J. of Antibiotics. October 2000, Vol. 53. No. 10, pages 1145-1153, entire document.	1, 4-13, 15-20, 26-28, 32, 34-37 — 14
X	US 4,732,680 A (WEAVER et al.) 22 March 1988 (22.03.1988), columns 5-6.	1

Form PCT/ISA/210 (continuation of second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/40398

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1, 4-25 (in-part) 30, 32, 34-37 (in-part), 40, 41

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/40398

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1, 4-30, 32, 34-37, 40, and 41, drawn to methods for improving a production level of a secondary metabolite.

Group II, claim(s) 2, 4-25, 33-37, and 42-44, drawn to methods for enhancing a component profile of secondary metabolites.

Group III, claim(s) 3-25, 31, and 45-46, drawn to methods for identifying a novel secondary metabolite.

Group IV, claim(s) 38, drawn to methods for isolating a novel secondary metabolite.

Group V, claim(s) 39, drawn to methods for detection of secondary metabolites.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The technical feature of Group I is the method steps in the method for improving a production level of a secondary metabolite; however, this technical feature is NOT a special technical feature since said feature does not define a contribution of the claimed invention over the prior art. Claim 1 broadly reads on, for example, *any* bioconversion using organisms and an organic medium supplement. The art is replete with examples, such as in U.S. Patent Number 4,732,680 which teaches methods of bioconversion by adding an organic compound to the microbial population (see column 5, lines 19-23).

Since Group I lacks a special technical feature, all other categories of inventions, namely the additional, different methods as delineated by Groups II-V, cannot share a special technical feature with Group I and, thus, do not have unity of invention with Group I.

Continuation of B. FIELDS SEARCHED Item 3: STN (CAPLUS) search terms: DMSO, EtOH, dimethylsulfone, tetracenomycin, chloramphenicol, thiostrepton, taxol, culture, metabolite, microorganism, lichen, plant, fungus